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STUDIES OF THE EFFECTS OF ANTINUCLEAR ANTIBODIES ON PROTEIN SYNTHESIS.

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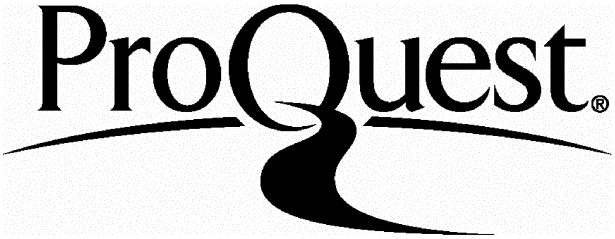

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Summary.

1. Undegraded and active poly(A)$^+$RNAs were obtained from tissues (rat liver or calf thymus) and cell lines (K562, NS 1 or HMy2) using sucrose gradient ultracentrifugation, guanidinium thiocyanate and rapid phenol-chloroform deproteinization. The above methods yielded 2-3% of total RNA which was as mRNA, found to be an undegraded product by analysis on either PAG or agarose gel electrophoresis. Single cycle oligo(dT) cellulose chromatography was used to purify poly(A)$^+$RNA and the percentage yield was 0.6-4% of total RNA. These poly(A)$^+$RNAs were in fact active templates and gave more than 3 fold increase in the incorporation of radioactivity over the control (without exogenous mRNA) in the rabbit reticulocyte lysate cell-free system. The fidelity of protein synthesis could be shown by gel electrophoresis. The incorporation of radioactivity was directly dependent on a concentration of added poly(A)$^+$RNA.

2. Two types of cell-free systems; wheat germ lysate and rabbit reticulocyte lysate, were studied. Both cell-free systems were shown to have high efficiency in translation of various types of RNA (TMV RNA, globin mRNA, polu U and poly(A)$^+$RNA from rat liver, K562 and NS 1 cells). The rabbit reticulocyte lysate was more efficient than wheat germ lysate in the synthesis of high M.W. protein (TMV protein at M.W. of 176K) but less sensitive to salt concentration. TMV RNA was the most active template since its rate of incorporation of radioactivity was approximately 100 fold above the control (without exogenous mRNA).

3. In studies of the effects of ANAs on protein synthesis in cell-free systems, either rabbit reticulocyte lysate or wheat germ lysate was used to synthesize protein directed by TMV RNA, globin mRNA, poly U or poly(A)$^+$RNA from K562 and NS 1 cells. IgG fractions (36 samples from 8 subgroups of ANAs) prepared from the sera of SLE patients were examined and their effects detected by comparing the incorporation of radioactivity with normal IgG controls. The translation product was further characterized using a single-dimensional gel electrophoresis technique.

It was demonstrated that some of these ANA samples had an inhibitory effect on protein synthesis and that the inhibition was not specific to any particular group of ANA. The ability to inhibit protein synthesis by different ANAs was different, since the percentage inhibition varied from 3% to 96%. However, the inhibition was dose-dependent in each case (IgG concentration). These ANA samples inhibited mRNA-directed protein synthesis in either rabbit reticulocyte lysate or wheat germ lysate cell-free systems.

ANAs did not appear to inhibit translation preferentially on any particular type of messenger. Messengers from various sources were affected by these ANAs with only slight difference in the percentage inhibition. The results of gel analysis demonstrated that the amount of protein synthesized was dependent on percentage inhibition and the inhibition was not related to any specific type of protein. At high percentage inhibition, there was no protein synthesis and synthesis of low M.W. protein was seen in the samples that exhibited low percentage inhibition.

The mechanism of protein synthesis inhibition by different ANAs appeared to be different. AntiRo (Frayne) and antiDNA (Jonas),

which showed high percentage inhibition (more than 90%), exerted the effect of inhibition at the level of elongation, whereas, other ANAs with inhibition between 20% and 40% blocked protein synthesis at the initiation step.

The above results clearly showed that some ANA samples had inhibitory effects, on protein synthesis in cell-free systems. The significance of the inhibition in these systems by ANAs was unclear.

4. The effects of ANAs on protein synthesis were further examined in intact cells. Several techniques were employed to transfer IgG molecules into cells, including fusion with liposomes or red cell ghosts, transfer via Fcγ receptors on cells, and permeabilization of cells in the presence of ANA. This study indicated that there was a limitation in transfer of macromolecules into cells. The protein synthesis pattern of cell lines was more complicated than protein synthesized in cell-free systems. Owing to the limitation involved, the ANAs did not show any effects on protein synthesis in the intact cells.

Abbreviations.

A_{280}	-	Absorbance at 280nm
ANA	-	Antinuclear antibody
BSA	-	Bovine serum albumin
DMSO	-	Dimethyl sulfoxide
ds-DNA	-	Double-stranded deoxyribonucleic acid
DTT	-	dithiotritol
ELISA	-	Enzyme linked immunosorbent assay
HEPES	-	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HnRNA	-	Heterogeneous nuclear ribonucleic acid
Ig	-	Immunoglobulin
K	-	Kilodalton
MCTD	-	Mixed connective tissue disease
M.W.	-	Molecular weight
mRNA	-	Messenger ribonucleic acid
Oligo (dT)	-	Oligothymidylic acid
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffer saline
pI	-	Isoelectric point
poly A	-	Polyadenylic acid
poly U	-	Polyuridylic acid
PPO	-	2,5-diphenyloxazole
Rf	-	Relative mobility
RNase	-	Ribonuclease
rRNA	-	Ribosomal ribonucleic acid
SDS	-	Sodium dodecylsulphate
SLE	-	Systemic lupus erythematosus
snRNP	-	Small nuclear ribonucleoprotein
ss-DNA	-	Single-stranded deoxyribonucleic acid
TEMED	-	Tetramethyl ethylene diamine
TMV RNA	-	Tobacco mosaic virus ribonucleic acid
tRNA	-	Transfer ribonucleic acid
Tween 20	-	Polyoxyethylene sorbitan monolaurate

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1. Introduction.

1.1. General Introduction.

It is now well established that RNA mediates the expression of DNA information in living cells. In the nucleus, RNA is synthesized on a DNA template by a process called transcription. All forms of eukaryotic primary transcripts (RNAs) are processed to form shorter, native forms of RNA with precise biological functions. The mature RNAs are transported into the cytoplasm for use in synthesis of proteins (translation process). Protein synthesis involves three types of RNA; mRNA, rRNA and tRNA.

The heterogeneous giant RNA molecule; called heterogeneous nuclear RNA (hnRNA) is found in the nucleus as a nucleoprotein particle; hnRNP. This hnRNA has been shown to be the precursor of mRNA by analysis of its nucleotide sequence. The actual model of the hnRNA is not known yet. The structure and function of the hnRNP are briefly described in section 1.2. The mature mRNA results from post transcriptional processes which occur in the nucleus. These processes include RNA splicing or processing, addition of polyA at the 3' terminal and methylation (in section 1.3). The exact mechanism of splicing and the enzymes which take part in this process are not known yet. There are a number of possible hypotheses about this mechanism (in section 1.4). Some evidence suggests the involvement of snRNA in this process. The snRNAs are found both in hnRNP complex and free in the nucleus and their functions are not exactly known yet (in section 1.5).

The translation process occurs in the cytoplasm and involves a great number of chemical reactions. The mechanism of the individual steps in translation in both prokaryotes and eukaryotes, including the control and inhibition of eukaryotic trans-

lation system are briefly reviewed in section 1.6.

The snRNAs are recognized by antibodies (antinuclear antibodies, ANAs) in sera of patients with autoimmune disease; MCTD and SLE. Some evidence suggests that these ANAs might be involved in the splicing process, transcription and translation. Therefore, the effect of ANAs on protein synthesis is studied in this thesis. The disease SLE, together with the incidence and specificity of ANAs are briefly reviewed in section 1.7.

1.2. Structure and Function of HnRNP.

In 1961, Georgiev found that there are two classes of RNA in the nuclei of eukaryotes; rRNA with base composition rich in G+C and RNA with DNA-like base composition, which is heterogeneous in size; dRNA. Since then, the existence, structural organization and function of this nuclear dDNA have been studied in many eukaryotic systems. Various terms are designated for this nuclear dDNA which are dependent on its structure and function; nuclear AU-rich RNA or dRNA; heterogeneous , heterodisperse RNA or hnRNA; messenger like or HlRNA and precursor of mRNA or pre-mRNA. HnRNA and pre-mRNA are in common use. This hnRNA is characterized by a low G+C content, which resembles total cellular DNA. It is heterogeneous with high M.W. and possesses a very high sedimentation coefficient in the range of 70-100 S (Georgiev et al., 1972). Samarina (1964) proposed that this hnRNA was a precursor of cytoplasmic mRNA, synthesized in nucleus with a high rate and cleaved into shorter chain by a nuclear processing mechanism. Therefore, this hnRNA is designated as pre-mRNA.

1.2.1. Structure and Characteristics of HnRNP

1.2.1.a. The structure of hnRNP under electron microscope.

The 30S particles, consisting of pre-mRNA complex with protein, have been isolated from the nuclei of rat liver or Ehrlich ascites carcinoma cells and they look like homogeneous globules about 200A° in diameter under electron microscopy (Samarina et al., 1968). The 30S particles are the monomers of the bigger complexes that are organized like the polysome. These large complexes are heterogeneous particles with sedimentation coefficient from 30-400S, containing high M.W. pre-mRNA and designated as polyparticles. These polyparticles consist of the same components as 30S particles; pre-mRNA and a major protein with a M.W. of 40K (calls "informatin"), and are converted into 30S particle when treated with a mild RNase. In 1974, Georgiev had analyzed the hnRNP by electron microscope and revealed a variety of shapes and sizes rather than a homogeneous structure as in early work. They are not well visualized in either negative or positive staining (Malcolm and Sommerville, 1977; Martin et al., 1978) and show a fibrilar configuration in the pellet which is obtained after centrifugation (Malcolm and Sommerville, 1977).. The nRNP complexes have the appearance of a tangled string of bead-like structure. Since a low concentration of RNase rapidly causes random dispersion of single beads, it is assumed that the tangled string is hnRNA and RNA may maintain the structure of the bead itself. However, its disintegration by RNase digestion can be due to the combined effect of proteolysis and nucleolysis.

1.2.1.b. Characteristics of the hnRNP units.

According to the sensitivity of hnRNP to salt and RNase,

hnRNP in eukaryotes can be separated into two classes of units; monoparticles and heterogeneous complexes or polyparticles (Jacob et al., 1981).

i). Monoparticles.

Monoparticle is found in 30-50S range after digestion of hnRNP with RNase under mild conditions. Its sedimentation coefficient is heterogeneous and this is not due to hydrolysis of a large monoparticle nor due to the existence of two or more classes of monoparticle. Monoparticle is also found to be heterogeneous in shape; ellipsoid, triangles and rectangles are observed under electron microscope. The size heterogeneity of monoparticle does not result from a set of protein at M.W. of 28K, 41K, 43K, and 51K which are present in small amount. (Stévenin et al., 1977).

When monoparticles are treated with medium RNase concentration, a substantial amount of RNA is hydrolyzed and certain proteins are released from monoparticles (Stévenin et al., 1977 and 1979). The protein of the residual RNA and the released protein are different and designated as α and β proteins, respectively. The α proteins are a set of 8-10 polypeptides with a M.W. range between 28K and 38K and have pI values of 5-9 while the β proteins comprise a few basic polypeptides with M.W. of 28K-38K and 6 major polypeptides of M.W. 41K, 43K, 51K, 55K, 62K and 108K and their pI's are 5-7. The distribution of α and β proteins in monoparticle is 40% and 60% of total monoparticle protein, respectively. There are three hypotheses for the model structure of monoparticles; A, B, and C. Model A suggests the existence of 2 classes of monoparticles M_α and M_β with different RNase sensitivity and heterogeneous in size. Model B suggests the existence of

only one class of monoparticle with a core of α protein surrounded by β protein while in the model C, the α and β proteins are distributed in a random way. (Stévenin et al., 1977, Gattoni et al., 1978).

ii). Heterogeneous complex, (HC).

HC is RNP with typical protein composition. It has a sedimentation coefficient in range of 50S to 200S and is a product of hnRNP after removal of monoparticle. The proteins are acidic with M.W. range of 23K to 200K and have pI of 5 to 7. HC differs from native hnRNP by having a low amount of protein below 40K (Stévenin et al., 1977; Fuchs and Jacob, 1979). The major proteins of HC are found between 60K and 80K and the protein with the M.W. of actin (42K)is enriched in this HC. The size of the RNA of the complex is 20-300 nucleotides, which is much smaller than that of hnRNP after isolation by salt treatment. This may be due to the RNase activity which was used to release the monoparticle. However, the HC is more resistant to RNase than the monoparticle and protein in the HC may protect the molecule from RNase (Stévenin et al., 1977).

When hnRNP is treated with NaCl, monoparticle proteins are released. The protein composition of the salt-resistant RNP is close to that of the HC while their hnRNA is much larger in size, up to 6000 nucleotides (Fuchs and Jacob, 1979). HC are also resistant to salt dissociation. Salt-resistant RNP contains snRNA in addition to hnRNA and it is still not known whether snRNA belongs to HC or monoparticle. The proteins in the range of 23K- 200K in hnRNP are phosphorylated in vivo. The most prominent phosphoproteins are found in the 28K-38K range and remain associated

with the HC or salt-resistant RNP. All proteins are detected in all fractions of HC with various sedimentation coefficient but their relative distributions are different. The HC are a population of hnRNP with gradual variation of protein content. The reason for this variation in protein distribution in HC is unknown but it is suggested that it might be involved in RNA processing and more evidence is needed to explain this role fully (Stévenin et al.,1977).

1.2.1.c. Model of hnRNP.

The configuration of hnRNA, protein and nuclear matrix are not well understood. Several models of hnRNP configuration have been postulated. All the models proposed employ the concept of a bead-on-a-string structure in which a string of RNA was localized on the surface of a globular protein complexes (the beads). The difference among the models are the protein composition and the presence of snRNA.

Samarina and Georgiev's group first postulated a polysome like structure in which the bead was made of 20-40 identical polypeptides (macroglobular particles; informofer), each having a homogeneous protein with a M.W. of 40K and called "informatin" Each informofer was combined with a part of RNA with M.W. of 200K (Samarina et al., 1968; Georgiev, 1974). Martin et al. (1978) had supported this model but with a slight modification, the linker was dsRNA from the observation that dsRNA region was not complexed with any protein at all in hnRNP. Calvet and Pederson (1978) and Le Stourgeon et al. (1978) postulated a stoichiometric interaction of 6 proteins as the bead structures. All of these models assume a regularly repeated subunit organization similar to ribosome in polysome.

From the existence of two classes of hnRNP population; monoparticle and HC (Jacob et al., 1981), and the association of hnRNP with nuclear matrix in vivo (Miller et al., 1978), other models of hnRNP have been proposed. Stévenin et al., (1977) suggested that the fibrils were joined by two classes of components the monoparticles and HC interspersed along the fibrils (Fuchs and Jacob, 1979). Jacob's group (1981) proposed 3 models; A, B, and C. Model A showed an alternation of the 2 classes of RNP along the fibril, while in model B, monoparticles were clustered at one end and HC were at the other end of the RNA molecule. Model C assumed that HC constituted an uninterrupted background structure. Some of the RNA sequences are within that structure or tightly bound to it and alternate with sequences emerging out of it and cover with monoparticle proteins. From the RNase and NaCl treatment experiments and the existence of nuclear matrix which are associated with hnRNP, model C is the most plausible model for hnRNP but at the present all the models are still compatible with experimental evidence.

These models need to be re-examined after the discovery of non-coding sequences (introns) in hnRNA, snRNA-hnRNA hybridization and the splicing mechanism. The size and number of introns may play a role in specifying the secondary and tertiary structure of hnRNA which in turn must define configuration of hnRNA-protein complex. The hnRNA is a very unstable molecule and interacts with protein and other nuclear components. This property contrasts with the more permanent and regular nucleic acid-protein interactions characteristic of chromatin and ribosomes. The polysomal-like structure should be reconsidered.

1.2.2. Constituent and Function of HnRNP.

1.2.2.a. HnRNA.

Characterization of both chemical structure and properties of hnRNA and its relation to cytoplasmic mRNA have been studied in hnRNP and in both units; monoparticle and polyparticle (see Perry, 1976; Van Venrooij and Janssen,1978; Sekeris and Guialis,1981; Samarina and Krichevskaya, 1981 for review). These hnRNAs which are extracted from nRNP particles are heterogeneous in size, comprised of DNA-like base, have high rate of turnover, contain polyA tract at the 3' terminal and cap structure at the 5' terminal and have a ds-RNA sequence or ds hairpin structure.

Monoparticles have been shown to contain mRNA sequences by hybridization between RNA isolated from these particles and cDNA. Moreover, the results from experiments of hnRNA translation in cell-free systems, transcription kinetics and the existence of pre-mRNA (such as globin mRNA) have indicated that mRNA sequences are found in hnRNA molecules in both monoparticle and polyparticle.It has been observed that 85% of the cytoplasmic poly(A)$^+$RNA have counterparts in monoparticles and only 5-10% of this RNA is transferred to cytoplasm as mRNA (Kinniburgh and Martin, 1976). Most of the RNA is located in the nuclei and this means that the greater part of hnRNA molecules turnover within the nucleus. The hnRNA is degraded to different degrees and the size of the hnRNA can be determined under non-denaturing condition. Economidis and Pederson (1982) demonstrated that the hnRNP which is transcribed in vitro had the same properties as the nuclear RNP particle in which hnRNA was found in vivo. It is assumed that small amounts of RNA in polyparticle and monoparticle contained mRNA sequences.

The polyA sequences, characteristic of most cytoplasmic mRNAs, have been shown to be present in hnRNA and hnRNP complex in polyparticle. (see Samarina and Krichevskaya, 1981 for review). These polyA sequences were tightly bound to protein in the form of low M.W. nuclear entities of about 15S fraction from sucrose gradient and showed a density of 1.24-1.26 gm/ml in $CsSO_4$ gradient.PolyA RNP particles are also found in polysomal cytoplasmic RNA and have a sedimentation coefficient of 14S. Their polyA RNAs estimated to be 200-230 nucleotides of which most residues are adenosine (88%). The size and base composition of the RNA from nuclear RNP appear to be similar to that of cytoplasmic RNA. These polyA are not transcribed from DNA but are added into hnRNA during a post transcriptional process. Protein composition of poly A RNP particles isdistinct from that of 30S monoparticle but the number of polypeptides is not yet clear. However, many reports have suggested that a major protein at M.W. between 70K and 86K is tightly bound to a polyA sequence, which other proteins are less tightly bound to polyA (Quinlan et al., 1977). These polyA may play a role in the normal maturation of mRNA and transport of mRNA to cytoplasm.

Short oligo(A) sequences(20-40 nucleotides) are also found in hnRNA from both monoparticles and polyparticles at the 3' end. These oligomers are located inside the molecule and transcribed from DNA (Edmonds et al., 1976). It is suggested that these sequences might be a primer for binding of long chain polyA in post-transcriptional process. Only crude 15S RNAs contain oligo(U) sequences(15-50 nucleotides). These sequences are localized near the 5' end and hydrogen -bonded to oligo(A) to polyA sequence in hnRNP structure (Kish and Pederson, 1977).

The stability of these oligo(U)-polyA duplex may be related to protein which is bound to polyA sequence. The function of this duplex remains unclear. It may be responsible for maintenance of the pre-mRNA secondary structure which in turn may be related to pre-RNA processing and transport.

The pre-mRNAs of nuclear RNP particles also contain triphosphorylated nucleotides as well as cap structure at the 5' end.

Most, if not all, hnRNA molecules from eukaryotic cells have a stable hairpin structure which includes an intramolecular ds region (see Naora, 1979 for review). These structures are RNase-resistant and vary in length from 10-20 to several hundred base pairs. Two classes of ds sequences have been revealed in hnRNA;

i). More stable ds sequence, which are resistant to RNase A and T_1, and which comprise 1-1.1% of the nucleotidesin hnRNA from HeLa cells.

ii). Less stable ds sequences, which comprise an additional 1.5-2% of the nucleotidesin hnRNA, and are sensitive to RNase III and are not resistant to RNase A and T_1 unless the salt concentration is raised to 0.63M.

The proportion of ds sequences in the pre-mRNA of 30S particles is about 6%, only one ds sequence containing 34-38 nucleotides with average size of approximately 50A° may exist in each 30S monoparticle. Therefore, most of the pre-mRNAs in 30S particles is in single-stranded form. The ds structures are localized on the surface of the particle and are almost free of protein. Biochemical and physical data suggest that pre-mRNA is already complexed with proteins during its synthesis and this

binding prevents the formation of secondary structure of pre-mRNA. Naora et al. (1979) have shown that parts of the nucleotide sequence present in the 5'-and 3'-terminal regions of many cytoplasmic mRNAs are derived from ds hairpin structure of hnRNA (pre-mRNA). They also suggested that ds sequences might play a possible role in the processing of non-coding sequences of pre-mRNA. Ds regions in hnRNA can result from intramolecular as well as intermolecular interaction. Intramolecular ds sequences can be formed by base pairs of contiguous (hairpin) and long range (loops) complementary sequences while intermolecular interaction can occur between two hnRNA molecules and between hnRNA and snRNA.

1.2.2.b. SnRNA.

There are many species of snRNA which are detected in cell nuclei and cytoplasm. They contain 90-200 nucleotides, have sedimentation coefficient in the 4-8S range and consist of modified nucleotides. They may have different functions in the cell dependent upon their contents, localization and metabolic activity. However, their roles remain unclear. The structure and function of these snRNA species will be described later in more detail in section 1.5.

Of the snRNA detected in nuclei, some are hnRNA constituents due to hydrogen-bond (of about 10-25 base pairs)formation to hnRNA (see Jacob et al., 1981; Sekeris and Guialis, 1981; Samarina and Krichevskaya, 1981 for review). The direct association of snRNA to hnRNA is found in both momoparticles and HC and represents about 6% of the hnRNA. Therefore, a functional role for the snRNA seems more likely than a structural role. The

function of the association of snRNA with hnRNA is not clear yet. It is suggested that snRNA might participate in structural organization of the nuclear RNP particle. SnRNA species in both monoparticlesand HC appear to be similar and independent of the particle size. Comparison of the proteins is difficult because their concentrations are not certainly determined. SnRNA protein consists of polypeptides with M.W. between 10K and 70K. The snRNA species does not distribute equally between hnRNP of various sizes. The U1, U2 and one of the three 4.5S RNA are found to complex with nuclear RNP particles. In monoparticles, U1 RNA is the most prominent snRNA while the amounts of U1 and U2 are equivalent in larger hnRNP. The high specificity of the protein bound to snRNA and the interaction of U1 RNA to pre-mRNA at specific region may be responsible for the splicing of pre-mRNA.

1.2.2.c. Proteins.

The protein composition of intact hnRNP particles is very complex, consisting of a series of proteins ranging in M.W. from 23K to 200K with some predominant components around 40K (Gallinaro-Matringe and Jacob,1973). This 40K protein is most probably identical with the protein that is the structural components of the informatin (Samarina et al., 1968) or informofer (an aggregated form of 20-40 informatin molecules, Lukanidin et al., 1972) or high salt washed of 30S particles. There is disagreement over the number of different protein species involved in particle formation. Northemann et al. (1978) reported that the difference in the protein composition of hnRNP particles might be the direct result of differences in the isolation procedure. Sonication of nuclei and salt concentration in buffer destroys chromatin and

nuclear matrix and may lead to redistribution of their components. HnRNP proteins may be classified according to their distribution between monoparticles and polyparticles. The protein composition of monoparticles is quite simple. For example, in the HeLa cells these proteins are basic polypeptides with high glycine content and dimethylarginine which may be involved in the interaction between protein and nucleic acid (Samarina and Krichevskaya, 1981). Isolation of the protein of polyparticles, which is tightly bound to RNA, has not started yet.

Irrespective to the tissue or cell type, and even the presence of protease inhibitors, the most prominent protein in hnRNP particle are in the M.W. range of 30K to 40K (Pederson, 1980). There is disagreement over their proportions, for example in the HeLa cells, they constitute 26% of the protein mass of nRNP while they represent 50% of the total nRNP protein in brain nuclei (Gallinaro-Matringe et al., 1975). It is generally agreed that this set of proteins can be removed at high salt concentration (Faiferman and Pogo, 1975; Beyer et al., 1977). Some salt labile proteins are basic, with pI of 8-9, have an unusual amino acid composition including high glycine content(25%),high hydrophobic amino acid content and a modified basic residue, N^6N^6-dimethylarginine (Beyer et al., 1977).

SDS-PAGE is the most effective method for investigation of heterogeneity of the proteins associated with hnRNA. It has been demonstrated between 6 and 50 protein species (Northemann et al., 1978; Brunell and Lelay, 1979). Some of the proteins associated with hnRNA maybe species-specific, such as protein (70-80K) which is associated with the polyA of hnRNP particles (Quinlan et al.,1977). The problems in study of the protein comp-

osition of hnRNP complex are the purity and the presence of secondary and tertiary structure of hnRNA.

1.3. Maturation and Structure of mRNA.

Recent studies of mRNA have demonstrated that most eukaryotic mRNAs have a unique terminal structure at the 5' terminus and a polyA sequence at the 3' terminus (Lodish, 1976). The mRNA is transcribed from DNA as a pre-mRNA which contains non-coding sequences(or introns) and coding sequences or (exons).There are post-transcriptional modifications of mRNA, including polyA addition, RNA splicing, capping and methylation which occurs in the nucleus and results in the formation of mature mRNAs. These mature mRNAs are transported into the cytoplasm and some of them are bound to ribosomes and direct the synthesis of polypeptide chains.

1.3.3. Post-transcriptional Modification of mRNA.

1.3.1.a. PolyA addition.

Most eukaryotic mRNAs and viral mRNAs contain a polyA sequence, about 50-200 residues,at the 3' end.PolyA addition is a rapid process and precedes splicing of RNA as shown by kinetic labelling and determination of the size of poly(A)$^+$RNA (Nevins and Darnell, 1978; Abelson, 1979). There are two processes of polyadenylation; the first process is a step-wise process in which one adenylic residue is added to nuclear RNA at a time by polyA polymerase (Lewin, 1980) and the second process involves two steps, cleavage and polyA addition (Nevins and Darnell, 1978; Nevins et al., 1980; Hofer and Darnell, 1981). In the second process, it is possible that the transcript is cleaved by an endonuclease and and there is a specific site in the structure of nucleic acid for

the recognition of this enzyme.

The hexanucleotide AAUAAA sequence in the 3' noncoding region of mRNA is highly conserved, therefore, it is thought to play a role in formation of a recognition site for cleavage or in recognition by the polyA polymerase (see for review Nevins, 1983; Baralle,1983). This sequence (AAUAAA) is present in many mRNAs about 10-30 residues away from the 3' terminal polyA sequence. The 3' noncoding sequences have been found to vary in length and are not essential to mRNA function. The role of the AAUAAA sequence in polyadenylation has been investigated both in vitro and in vivo. By study of deletion mutants of SV40 at or around the AAUAAA sequence, it was found that the polyadenylation site was moved dependent on the size of deletion if deletion occurred within 10-19 nucleotide range from the AAUAAA sequence and stopped if the deletion included the AAUAAA sequence. From these results, the sequence AAUAAA was essential for the formation of poly(A)$^+$RNA and it was suggested that this sequence could specify the proper cleavage of the RNA chain by acting as a part of recognition site. This was confirmed by a recent study of adenovirus AAUAAA mutation, only 10% of the normal amount of polyA RNA is produced, if the AAUAAA is converted to AAGAAA (Montell et al., 1983). Therefore, AAUAAA sequence is required for the cleavage step preceding polyadenylation to occur efficiently but it is not required for RNA polyadenylation.

This AAUAAA may not be the recognition site for all polyadenylation. There is heterogeneity in the site of polyA addition in vivo. For example, in bovine prolactin mRNA polyA appears to be added at several sites within a 10 base pair region and the adenovirus 3 transcription unit has 2 polyA sites. Moreover,

some picornaviruses and polyovirus, which completely lack of the AAUAAA sequence, can also form polyadenylated mRNA. Different polyadenylation mechanisms may exist. However, the precise mechanism of polyA addition is still unclear.

1.3.1.b. RNA splicing.

It is now accepted that many cellular and viral mRNA molecules are derived from high M.W.precursor (pre-mRNA). The coding sequences (exons) of many eukaryotic pre-mRNAs are interrupted by stretches of noncoding sequence (intron) of various size. The introns are deleted and the exons are then covalently joined by intramolecular ligation. This cleavage-ligation process is termed RNA splicing. This hypothesis requires a specific mechanism for selecting splice sites and a splicing process, involving enzymes for cleavage and ligation of the RNA molecule. The actual mechanism of the splicing process is still unclear. The details of RNA splicing will be described in section 1.4.

1.3.1.c. Capping and Methylation.

Many eukaryotic, viral mRNA and nuclear pre-mRNA contain a rather unusual structure called the "cap" 7-methylguanosine (m^7G) (Shatkin, 1976; Lewin, 1980). In general, the 5'-5' pyrophospate bridge is formed by guanylyl transferase and their terminal guanosine is methylated at the N^7 position. Sometime, the nucleotide at the 5' end of the transcript is methylated in the ribose moiety before linking to the m^7G. The formation of a capped terminus is a very early event in mRNA formation, since a capped-premature terminated transcript and a capped nascent chain less than 100 nucleotide long have been found (Babich et al., 1980). Shatkin (1976)

demonstrated that in vaccinia virus and HeLa cell, capping occurred after the initiation of transcription. In recent studies of the structure and function of mRNA transcripts, the"TATA" box, which was a strongly homology region and almost universally conserved, has been found. This "TATA" box is located approximately 30 nucleotides upstream from the capping site. These results lead to the speculation that this "TATA" box might have a role in the initiation of transcription in eukaryotes (Pribnow, 1979). In vivo, Baker et al. (1979) found that "TATA" box was not essential for transcription of certain viral mRNA and after a point mutation in "TATA" box (TAGA) the site of RNA synthesis was not changed while the rate of transcription was decreased. (Grosschedl et al., 1981). This "TATA" box is also required to guide the RNA polymerase into a correct initiation frame (Benoist and Chambon, 1981). The rapid addition of the cap and polyA to pre-mRNA tends to suggest that the 5' cap structure and 3' polyA sequence are involved in the protection of pre-mRNA from nuclease during RNA processing.

Methylation of mRNAs also occurs at internal adenylate residues at the N^6 position (Shatkin, 1976). The m^6A residues are added to the sequence in primary transcription and are conserved during RNA processing. Stoltzfus and Dane (1982) found that the formation of mature snRNA was reduced and the synthesis of protein from pre-mRNA was also increased when viral infected cells were incubated with cycloleucin, a drug that reduced the frequency of m^6A in retrovirus RNA. From these results it was suggested that m^6A residues might play a positive role in splicing, but more direct experiments are needed to prove this point.

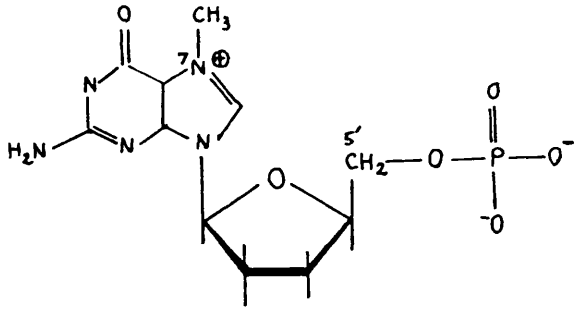
1.3.2. Structure and Function of Cap structure and PolyA.

1.3.2.a. Cap structure.

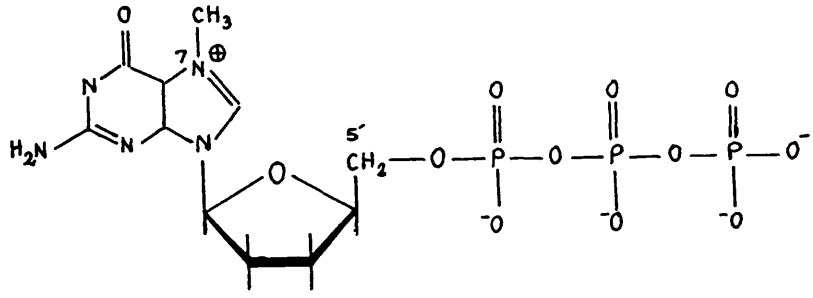
The cap, m^7G, at the 5' end of the transcript has been shown to have many functions (see Ravel and Groner, 1978; Baralle, 1983 for review). There are three types of cap; cap 0 (m^7GpppX) which has only one methylation at G, cap I (m^7GpppX^m) where the 3'-5' bond between the 2'-omethylated nucleotide and the next residue in the mRNA chain is stabilized against hydrolysis by alkali or T_2RNase because formation of the 2'-3'-cyclic intermediate required for cleavage is blocked and cap II ($m^7GpppX^mpX^m$) which has many methylations at the 5' terminal of mRNA.

The exact role of this structure in mRNA function has not yet been established. However, a number of functions have been proposed for this cap structure and two suggestions are more likely to be its function. The first one is to protect the mRNA molecule from degradation by phosphatase and nuclease, at the 5' terminus, and the second is to play an important role as a recognition signal in the initiation step of capped mRNA translation, by helping in the ribosome binding process or in specific protein binding. Other functions have been speculated including participation in mRNA processing and transport and involvement in the switching on or off of protein synthesis in the cell, but there is not enough evidence to support these functions.

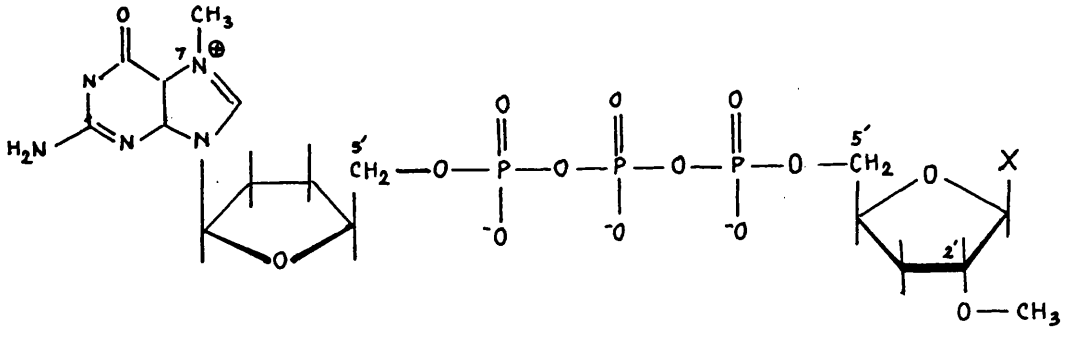
The stabilizing effect of the cap structure on mRNA has been examined by incubation of capped and uncapped mRNA in cell-free systems (Xenopus Oocyte, wheat germ lysate or L cell extract) and determination of mRNA stability. The results showed that mRNAs containing m^7GpppG^m or GpppG at their 5'termini were degraded more slowly than uncapped mRNA. This indicates that



7-methyl-guanosine-5'-monophosphate (m^7Gp)



7-methyl-guanosine-5'-triphosphate (m^7Gppp)



7-methyl-guanosine-5',5'-triphospho-2'-methyl-X ($m^7GpppXm$)

Fig. 1.1. Structure of cap analogues.

the 5' - 5' pyrophosphate bridge was sufficient for protection of the mRNA against nucleases. But this structure is not the only factor determining mRNA stability since uncapped natural mRNAs, such as several viral RNAs, are active and stable in in vivo and in vitro. It is assumed that mRNA is rapidly degraded after removal of the cap.

Concerning the role of the cap structure in translation, Kozak (1978) has suggested that the cap is used as a guide for the binding of the ribosome to mRNA. This suggestion is supported by in vivo and in vitro studies of mRNA translation and use of cap analogues such as m^7Gp, m^7Gppp, $m^7GpppXm$ (Fig 1.1.). In vivo, only capped viral mRNA is associated with polysomes in VSV infected BHK cells. In vitro, capped mRNAs are much more active in translation than improperly capped mRNAs and removal of the 5' cap of mRNA prevents translation. In particular, the uncapped mRNA fails to bind to the 40S subunit. In addition, the translation of capped mRNA is strongly inhibited by cap analogues. This inhibition seems to be by interference at the binding of mRNA to the ribosome subunit and the binding of mRNA and of Met-$tRNA_f$ to initiation factors, eIF-2 and eIF-4B (Shafritz et al., 1976). This inhibition is reversed by adding eIF-2. However, there are uncapped mRNAs, such as poliovirus and encephalomyocarditis (EMC) virus, are very active templates for protein synthesis and are not inhibited by m^7Gp. Modification of cap structure in mRNA by chemical or enzymatic procedures was shown to involve in the translation capacity of mRNA. Efficiency in cell-free system is either retained or lowered and this reduction may be resulted from both decreased stability of mRNA and its lower activity in binding to the ribosome (Wodnar-Filipowicz et al, 1978).

It is not clear which features of the cap are required for the process of ribosome binding. However, methylation at position 7 of the G and the 5'-5' pyrophosphate are essential while the sequence adjacent to the cap structure is unnecessary for binding of 40S subunit.

The cap binding protein (CBP) which is isolated from rabbit reticulocyte and mouse L cell, specifically recognises the cap of several mRNAs and stimulates the translation of capped mRNA in cell-free systems. CBP might facilitate mRNA-ribosomal interactions by unwinding the 5' terminal secondary structure of eukaryotic mRNA (Sonenberg et al., 1979).

These results indicate that the cap structure of eukaryotic mRNAs may have an important function in the initiation of translation but the exact nature of this function is not clearly understood.

1.3.2.b. PolyA.

The polyA sequence at the 3' end of mRNA behaves as a relatively homogeneous component with a sediment coefficient of about 4S and an electrophoretic mobility at 7S RNA component in PAGE. This value indicates a size of about 200 AMP residues. The length of this sequence varies in mRNA, and lower eukaryotes seem to have shorter polyA sequences (Brawerman, 1974).

The function of the polyA sequence has been determined and three general hypotheses for polyA function have been suggested. These include a role in mRNA stability, in mRNA processing and transport and in protein synthesis (Baralle, 1983; Ravel and Groner 1978; Nevins, 1983).

Several studies suggested that the polyA segment played a role in determining mRNA stability. Globin mRNA with polyA tracts of approximately 30 nucleotides or more was found to be stable, whereas that with a shorter polyA tract was found to be unstable (Nudel et al., 1976). Histone mRNA, which lacks polyA, was found to degrade rapidly after injection into HeLa cells (Huez et al., 1977). The 3' polyA had an important function in mRNA stability and the minimum length of the polyA segment required for stability was found to be about 30 adenylate residues. The shortening of polyA segments does not appear to reduce the translational actvity of mRNA and it occurred in the cytoplasm. However, there were results which suggested that polyA did not regulate mRNA stability. This included experiments demonstrating that deadenylated forms of interferon and α -2U-globulin mRNA did not differ in stability from their adenylated forms when microinjected into Xenopus oocytes (Deshpande et al., 1979) and there was no correlation between the stability of a mRNA and the size of its polyA tail in 66 different Dictyosteliom mRNAs that possessed different lengths of polyA tracts (Palatnik et al., 1980).

A role for polyadenylation in mRNA processing or transport is unlikely, since a number of mammalian viruses that totally lack a nuclear component have a polyA on their mRNA. Recently, Zeevi et al., (1982) showed that a number of mRNAs which were naturally poly(A)$^-$ or which become poly(A)$^-$ in 3'-deoxyadenosine-treated cells, were efficiently transported to the cytoplasm and their nuclear RNAs were properly spliced.

The possibility function of a polyA in protein synthesis has been investigated. Many studies suggested that a polyA segment was not required for translation in a cell-free system, since

histone mRNA and deadenylated rabbit globin mRNA were actively translated. In addition, artificial deadenylation or blockage of the polyA tail with poly U did not significantly reduce the efficiency of mRNA translation (Munoz and Darnell, 1974). In contrast, Doel and Carey (1976) showed that native poly $(A)^+$ ovalbumin mRNA was translated more efficiently than deadenylated ovalbumin mRNA in reticulocyte lysate, but no difference was observed in less active wheat germ lysate. Deshpande et al, (1979) found that in Xenopus oocytes, α-2U-globulin mRNA with a short polyA tract was translated less efficiently than the same mRNA possessing a long polyA tract. During in vitro translation of mRNA, many polynucleotides, including polyA, can inhibit the initiation step in reticulocyte lysates and different polymers varied greatly in their inhibitory activity (Lodish and Nathan,1972). This result suggested that the inhibitory activity was due to an interaction between polymer and the ribosome, which resulted in an overall reduction in initiation efficiency. Jacobson and Favreau(1983) also suggested that the polyA tract of mRNA had a function in protein synthesis that was competitively inhibited by exogenously added polyA and proposed that polyA had a function in protein synthesis and its other role in mRNA stability was indirect.

1.3.3. mRNA Transport.

The mature mRNA is formed after post transcriptional processing and is transported to the cytoplasm as a nRNP particle (Spirin,1969) via the nuclear pores (Jacob and Danielli,1972). Only fully processed molecules enter the cytoplasm (Nevins,1979). There appears to be no cytoplasmic processing of RNA; furthermore, unspliced mRNA does not normally accumulate in the cytoplasm of

differentiated cells. Thus, it seems that a mechanism must exist for selective transport of RNA sequences from the nucleus to the cytoplasm. The transport of mRNA may be related to size, capping and polyadenylation of mRNA and its chemical components. Biochemical and ultrastructure studies (Faiferman and Pogo,1975; Herman et al., 1976; Agutter et al., 1979;Moffett and Webb, 1983) indicated that nuclear RNA is normally transported to the cytoplasm through the nuclear pores and this process requires many components. However, the regulation of this mechanism is still unclear.

The rate of nuclear-cytoplasmic transport of different mRNAs varies. Histone mRNA are transported immediately after synthesis (Bonaldo et al., 1979) while other mRNAs require more than 1 hr for processing and transport (Wilson et al., 1978). The regulation of this mechanism is not known but much evidence suggests that mechanisms must exist for the selection of mRNA sequence at the post-transcriptional nuclear level which include selective processing of the primary transcript, multiple splicing enzyme system , the absence and presence of processing enzymes specific for classes of mRNA and the selection of fully processed mRNA for transport to the cytoplasm (Crick,1979). In general, processing is obligatory for the transport of mRNA to the cytoplasm. Interference with RNA processing may result in the inhibition of mRNA migration to the cytoplasm. The actual mechanism still cannot be resolved.

1.4. The Possible Hypothesis in RNA Splicing.

RNA splicing, in which the noncoding sequence (intron) is removed from pre-mRNA and coding sequences (exons) are joined

together, appears to be one of the terminal events in the post transcriptional process of mRNA. This splicing process is relatively slow and occurs exclusively in the nucleus (Nevins,1979). Nevins (1979) has demonstrated that the splicing of adenovirus fiber mRNA requires approximately 20 min and splicing intermediates are obligatory precursors in the pathway to the final mRNA. Moreover, in the case of globin mRNA, intermediates in the splicing pathway have been found, showing that the introns in a transcript are removed in a series of steps (Kinniburgh and Ross, 1979).

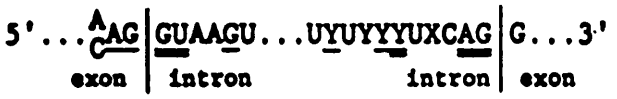
1.4.1. The Recognition of Splice Site.

The splice sites in nuclear mRNA are always marked by a sequence resemblance. The consensus sequences are:-

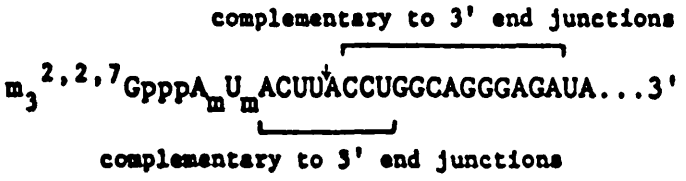
$5'\,{}^{A}_{C}AG/\underline{GT}{}^{A}_{G}AGT$ for the donor site and $\left({}^{T}_{C}\right)_n N^{C}_{T}\underline{AG}/GT^{G}_{T}\ 3'$ for the acceptor splice site and the first two GT and the last two AG nucleotides of an intron are strictly conserved. These results came from analysis of the sequence at over 130 splice junctions by Mount (1982). These conserved splice sites must be involved in the actual cleavage. This is confirmed by the study of human β-thalassemia, α-thalassemia and adenovirus EIA. Orkin et al. (1982) found that in the β°globin gene, which had an A to G change at the 5' splice site of the second intron (AG), this resulted in the inactivation of this splice site and a low level of β globin production. A similar result was shown in α-thalassemia, which had a mutation removing 5' splice site, which resulted in the use of an alternative site contained in the middle of the exon (Felber et al., 1982). Thus, this mRNA is not able to produce globin. Furthermore, with adenovirus EIA, modification

a

hnRNA concensus sequences:



U1 RNA:



b

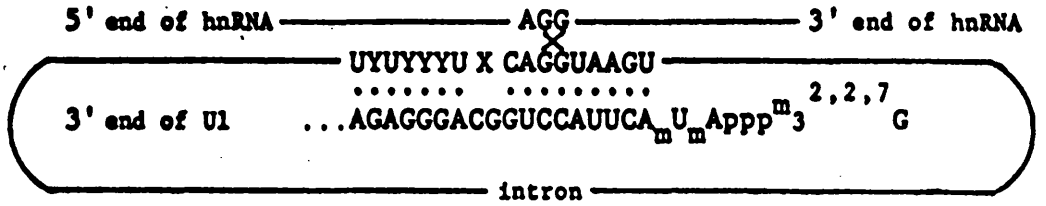


Fig. 1.2. Possible base pairing interaction between U1 RNA and splice junction consensus sequences in hnRNA.
a. Base sequences at the intron-exon junction of hnRNA and at the 5' end of U1 RNA.
b. A possible model of base pairing interactions between U1 RNA and sequences at both ends of an intron.

:from Lerner, et al.(1980) Nature, 283, 220

in the first splice site (GT) by conversion of a T to a G, inactivated the splice site and resulted in failure to produce mature EIA 12S mRNA (Montell et al., 1982). In contrast, deletion of portions of introns outside the consensus sequences generally does not have any effect, such as in rabbit globin gene, deletion leaving only 6 nucleotides of the intron next to the 5' splice site or 15 nucleotides next to the 3' splice site still allows accurate splicing (Kühn et al., 1983). From this evidence, the involvement of the consensus sequence in splice site selection is established.

SnRNPs have been proposed to be involved in hnRNA processing and in particular, in the splicing of pre-mRNA molecules (Abelson, 1979; Crick, 1979; Lerner et al., 1980). Lerner et al. (1980) have made a comparison of sequences of snRNA to known sequences of intron-exon splice junctions in many eukaryotic genes and found extensive complementarity between both the 5' and 3' end functions of intron-exon sequences and the sequences at the 5' terminal of U1 RNA. They have presented a model for possible base pairing interaction of U1 RNA and the splice junctions of hnRNA (Fig 1.2). A similar model was also reported by Roger and Wall (1980). Lerner et al. (1980) also showed that the intron-exon splice junctions were conserved through evolution and U1 RNA with the 5' end missing was not associated with hnRNA. These proposals have some evidence from in vitro experiments to support this role. Yang et al. (1981) found that addition of antiRNP-or antiSm-antiserum from SLE patients resulted in a failure to splice the adenovirus RNA transcript. In contrast, antiRo-and antiLa-antisera which are antibodies against cytoplasmic RNA and a separate class of nuclear RNP, respectively had no effect. In addition, Mount et al. (1983) also found that U1 RNP was selectively

bound to the 5' splice site of an artificial β-globin pre-mRNA. The model is not completed yet, an alternative splicing mechanism may exist. The results of Spritz et al. (1981)indicated that regions other than consensus regions of intron could affect processing. The possibility exists that the processing site and its interaction with snRNA might be affected by the secondary structures of the RNAs involved and all splice sites might not be identical. Naora and Deacon (1981) reported that a better match between U1 RNA and the splice junction of insulin pre-mRNA could be made with model for the secondary structure of U1 RNA which was demonstrated by Epstein et al. (1981) and the sequence of the exon and the intron portions of insulin pre-mRNA. Moreover, mitochondria have not been found to contain snRNA molecules but mitochondrial messengers are spliced. The hnRNP structure could bring certain 5' and 3' splice sites into proximity and lead to exposure of the consensus sequence for splicing (Kühn et al., 1983). Langford and Gallwitz (1983) have shown that an octanucleotide that occurs 20 - 25 nucleotides upstream of the 3' splice site in all yeast mRNA was also required for splicing in addition to the conserved sequence at the splice sites.

Therefore, the specificity of splicing may require a primary sequence and double helical regions in the RNA transcripts and also interaction of the transcript with protein by means of snRNA (U1.RNA) and sequences of the splice junctions. The exact mechanism for selective splicing is still unknown.

1.4.2. Mechanism of Splicing Reaction.

Abelson (1979) has pointed out the requirement of enzymes in splicing, which must accurately excise introns and then accurately

link the exons together. Multiple enzymes have been proposed for these processes (Crick, 1979). A variety of RNases are candidates for enzymes that specifically cleave hnRNA during its processing. Only RNase III, P , Q, (an exonuclease), T_2 and a few other enzymes exhibit specificity for tRNA precursor cleavage and for processing viral pre-mRNA. RNase III recognizes 16S rRNA and 23S rRNA of the 30S pre-rRNA and RNase P and RNase "E" recognize tRNA and 5S rRNA, respectively (Abelson, 1979). RNase III-like enzymes are present in eukaryotes, but their functions are not known (Abelson, 1979). The cleavage sites recognized by the RNases are dependent on RNA conformation, which is controlled by its nucleotide sequences and its associated protein. RNase P is an endonuclease which consists of a 20,000 M.W. basic protein and 300 nucleotide long RNA and pre-tRNA which generates the 5' termini of mature tRNA molecules. The enzyme recognizes the structural conformation of pre-tRNA molecules rather than nucleotide sequences (Kole and Altman, 1979).

No specific RNA cleavage enzyme or ligase has been found associated with U snRNP. The only example is RNase P, which is a small RNP, and is involved in processing of pre-tRNA. It is not known whether this RNase P is related to U snRNP particles. Therefore, the exact mechanism of splicing and the enzymes which take part in this process are not yet known.

1.5. Structure and Functions of SnRNA.

A special class of nuclear RNA with sedimentation coefficient ranging from 4 to 8S, referred as a low M.W. nuclear RNA or snRNA has been found in all eukaryotic cells and also in viruses and bacteria. Many species of these snRNAs are first discovered

in the nuclei and are later found in the cytoplasm. It appears that most of snRNA species are concentrated in the nucleus. However, some of them have been shown to shuttle between nucleus and cytoplasm. At first, these small RNA species were suspected to be the degradation products of higher M.W. RNA (hnRNA, rRNA and tRNA) because of the problem of digestion by endogenous RNase. Some of these problems have been overcome by using improved methods and RNase inhibitors or by finding a biological source with low RNase activity, such as Novikoff hepatoma cells. A class of RNA species with specific properties which represents approximately 0.5% of total cellular RNA has been found. Their structure and function are briefly described as follows (see Ro-Choi and Busch, 1974; Hellung-Larsen, 1977;Choi and Ro-Choi,1980; Reddy and Busch, 1981; Busch et al., 1982 for detail review).

i). About 100-300 nucleotide long.

ii). Rather stable with half-lives up to one cell cycle.

iii). Distributed in all cellular fractions including nucleolar, extranucleolar fraction, nucleus and cytoplasm.

iv). Some of them exist in RNP complex and hybridize with nuclear and nucleolar DNA.

v). Some of them have unusual distribution and content of modified nucleosides.

1.5.1. Classification and Structure of SnRNA.

These snRNAs are classified into many groups according to their size. They are isolated by sucrose gradient centrifugation and fractionated by electrophoresis or chromatography.

1.5.1.a. 4S RNA.

4S RNA is the only snRNA which is present in all subcellular fractions, with about 99% in the cytoplasm. In nucleolus, it has only 60% amino acid acceptor, contains a high amount of U and A and has less hydro-U and N^3-methyl-G than 4S RNA in the cytoplasm. It is suggested to be a precursor of nuclear and cytoplasmic tRNA. Nuclear 4S RNA has an aa.acceptor activity the same as that of cytoplasmic tRNA. It appears that the newly synthesized tRNA is converted to an active form as soon as it is formed. Cytoplasmic 4S RNAs are all tRNA's and have the typical clover leaf structure.

1.5.1.b. 4.5S RNA.

4.5S RNA is located in chromatin or nucleoplasm. It comprises three distinct molecular species; 4.5S RNA I, II, and III. 4.5S RNA I and II are 96 nucleotides long, contain approximately equal quantities of each of the four nucleotides and have no modified nucleotides. The base sequence at the 5'end is rich in purine and its nucleotide is 3',5' diphosphate G. At the 3' end it is rich in pyrimidine and U is the 3' terminal nucleotide. It has a high content of AMP residues at the center of the molecule. 4.5S RNA III is a unique RNA species with adenosine 3', 5'-diphosphate at the 5'-terminal and 2'-O-methyluridine at the 3'terminal. It contains ψ, N^2-methyl-G and N^6-methyl-A and has higher A and lower C content than 4.5 RNA I. Their functions are not known and they certainly are not tRNA precursors.

1.5.1.c. 5S RNA.

5S RNA has been found in both nucleolar and ribosomal RNA

fractions. There are three subspecies (I, II, and III) of nuclear 5S RNA and their functions are suggested to relate to the maturation process of the ribosome. It is clear that 5S RNA is a component of rRNA. It is associated with 28S rRNA by hydrogen bond Its synthesis is coordinated with 18S and 28S rRNA but their cistrons are located at different sites.

1.5.1.d. U RNA.

These snRNA have a high content of uridylic acid and consist of 6 major species; U1-U6. Most of them, except U3 RNA, are found in nucleoplasm as shown by immunofluorescence using specific antibodies directed against these RNPs. U3 RNA is found in the nucleolus. They are present in viruses and in prokaryotic and eukaryotic cells but their gel electrophoretic patterns are different. They are also found in all vertebrates and nonvertebrates. By sequences analysis, the snRNA's U1, U2, and U3 appear to have been conserved through evolution. Many reports showed that snRNA (U1-U6) are also present in cell as small RNP particles complexed with a set of polypeptides and these snRNA species are immunoprecipitated with a series of autoimmune sera from MCTD and SLE patients.

i). U1 RNA is found only in the nucleus and is the most abundant of the snRNA species. U1 RNA can be separated into two bands (U1a and U1b) with electrophoretic mobility lower than that of 5S RNA. The faster moving (U1a RNA) has a 3' terminal G and lacks a 5' terminal phosphate. Recently, only a single band of U1 RNA was found in HeLa cell and Novikoff hepatoma nuclei (Donis-Keller et al, 1977). However, mouse nuclei still contain two U1 RNA and two U1 RNA species from Erlich ascites

cells appear to be isomers. (Lerner and Steitz,1979).

ii). U2 RNA is localized in the extra nucleolar portion like U1 RNA. It contains a large number of modified nucleotides, including ten 2′-O-methyl nucleotides and ten ψ . Sequence analysis of U2 RNA confirmed that this RNA had one common 5' end with trimethyl G and two 3' end (A or C)(Shibata et al., 1975). This RNA was associated with protein as an RNP complex and was suggested to be involved in the synthesis of hnRNA. Its 3' end is protected by the secondary structure and attached protein.

iii). U3 RNA is specifically located in the nucleolus and is the first snRNA which is found to associate with precursor RNA. It is hydrogen bonded to nucleolar 28S and 32S rRNA along with 8S RNA and 5.5S RNA. It is capped with trimethyl G and can be separated into three subspecies (Reddy et al., 1979). Reddy et al. (1980) found that U3A and U3B differed by substitution of only 17 bases and had an identical length of 216 nucleotides. The ratio of U3 RNA/28S RNA is approximately 1/2, suggesting that only some nucleolar 28S RNAs are bound to these molecules. Since its association is only for a limited time, it may play a role in processing of nucleolar 28S RNA. The third species of U3 RNA has not been completely sequenced but it appears to be a minor variant of U3B RNA.

iv). U4 RNA is localized in the nucleoplasm and is also capped with trimethyl G. It has heterogeneity at position 97 (substitution of A → C). It contains a ψ near the 5' cap, 2′-O-methylated and m^6A (Reddy et al., 1981).

v). U5 RNA is the most enriched in uridine (37%) and is capped with trimethyl G (Choi and Ro-Choi, 1980).Like U4 RNA U5 RNA is homologous to U1 RNA. It was first called 5S RNA III and has at least two subspecies.

vi). U6 RNA is found to associate with perichromatin granules. Apart from U2 RNA it is the most highly modified, and in contrast to other UsnRNA, the modified nucleotides are in the center of the molecule (Epstein et al., 1980). U6 RNA is also capped at the 5' end, but their cap structure is not fully characterized. The cap structure is suggested to be XpppG and Xp is not a nucleotide (Epstein et al., 1980).

The nucleotide sequences of these U snRNA species have been determined. When the sequence of U3 RNA was compared with those of other snRNA species it was found that there were ten regions of homology in these molecules which varied in length, composition and spacing throughout the molecule. The areas of homology are at the 5' end, and also purine-rich and pyrimidine-rich sequences in various portions of the structure. Some of these variations may be related to the localization or to a flexibility for binding to various types of hnRNA. The longest homologous region is at position 1-26 (maybe up to 35) which has been implicated in hydrogen-bonding to the intron-exon junction.

1.5.2. Functions of SnRNA.

SnRNAs are present in all cellular fractions and represent only 0.5% of total cellular RNA. They may have an important role in the cell. However, their functions are unknown. By indirect evidence, some classes ofsnRNAs are implicated in a role such as

splicing of mRNA, transport of mRNA to ribosomes, translation regulation and stimulation of transcription.

1.5.2.a. A role of snRNA in RNA splicing.

U1 RNP has been proposed to be involved in splicing of pre-mRNA molecules as described in section 1.4.1. Other snRNA (U2, U4, U5, and U6) are also involved in processing of hnRNA since they are also associated with hnRNA. U6 RNA has been shown to be associated with perichromatin granules and contains different 5' cap which is not a nucleotide. Therefore, U6 RNA may have a different function from U2, U4 and U5 and it is possibly related to the formation of a precursor particle in the chromatin. (Daskal et al., 1980)

U3 RNA and 8S RNA are located in the nucleolus and they are hydrogen-bonded to precursor rRNP. It has been suggested that U3 RNA and 8S RNA might have complementarity to regions where specific cleavage of 45S or other rRNA precursors occurs to yield mature 5.8, 18 and 28S RNA's.. More evidence from sequencing of regions at the complementary site may confirm this function of U3 RNA and 8S RNA. Since there is good complementary between rat U3 RNA and Dictostelium D2 RNA and the exon region of a rRNA precursor of tetrahymena at the splice junction. Wise and Weiner (1980) proposed that U3 RNA and D2 RNA might be involved in splicing of ribosomal precursor RNA's.

1.5.2.b. A role of snRNA in translation and transcription.

SnRNAs have shown an inhibitory effect on translation of mRNA in cell-free systems as will be described in section 1.6.3. . The role of snRNA in gene transcription was studied in isolated

nuclei. Kanchisa et al. (1977) reported that nuclear 4.5S RNA could increase the number of binding sites for RNA polymerase to initiate transcription in calf thymus chromatin whereas Krause and Ringuette (1977) showed that a small RNA extracted from the chromatin of SV40-transformed WI38 human fibroblasts could stimulate transcription of normal WI38 nuclei to a level undistinguished from that of transformed nuclei. This snRNA contains loosely bound chromosomal protein (NHCP). The RNA,not the NHCP, was the active element stimulating transcription and the stimulatory effect of this RNA was found to be dose dependent. This RNA stimulated transcription by promoting new chain initiation. The active snRNA which showed the highest activity was in the region (290-320 nucleotides) from untreated RNA whereas urea-treated RNA showed the highest activity in region (160-175 nucleotides) in PAGE. Many studies suggested that the smaller species was a breakdown product of the larger one. The regulatory RNA may be heterogeneous. Ringuette et al. (1980) showed that snRNA from transformed human cells (SV40-WI38) could stimulate transcription in both human and monkey nuclei, although the stimulation observed in human nuclei was considerably greater. These results indicated that regulatory snRNAs were not unique to viral transformation and were tissue and species specific. Krause and Ringuette (1982) proposed that the active snRNA, acting in conjunction with nuclear protein, might help to destabilize the double helix in the controlling region of DNA, thereby facilitating entry of RNA polymerase I for the formation of an initiation complex.

Paoletti et al. (1980) found that some of the proteins in the loosely bound fraction of NHCP were tissue and species specific,

very heterogeneous in size and had intransient association with chromatin. This extract contains the high mobility group (HMG) protein and HMG 14 and 17 are associated with nucleosome core particles in the transcribable region of chromatin (Weisbrod and Weintraub, 1979). These results suggested that these proteins might have a role in selective gene regulation.

1.6. Translation Process.

1.6.1. The Mechanism of Translation System in Prokaryotes and Eukaryotes.

Translation consists of three major stages; initiation, elongation, and termination of the polypeptide chain. A common feature throughout the process is a high degree of specificity at every step, an association of many components and a requirement for energy. The brief review of translation process in both prokaryotes and eukaryotes is described as follows.

Most of the processes are better understood in a prokaryotic system. The initiation stage is a major step for control of the overall translation process. In 1968, Jacobson and Baltimore suggested that, in animals, several cellular mRNAs contain only one initiation site near to the 5' terminal (monocistronic mRNA) and code for only one protein. More recently, two different initiation sites have been detected on poliovirus RNA (Celma and Ehrenfeld, 1975) and many mRNAs are shown to contain several initiation sites. In all cases, however, only one of them, which is closest to the 5' end of mRNA, is active in vitro (Smith and Carrasco, 1978), while internal sites are inactivated by the secondary structure. In contrast to eukaryotes, many bacterial and bacteriophage mRNAs are polycistronic and each cistron has its

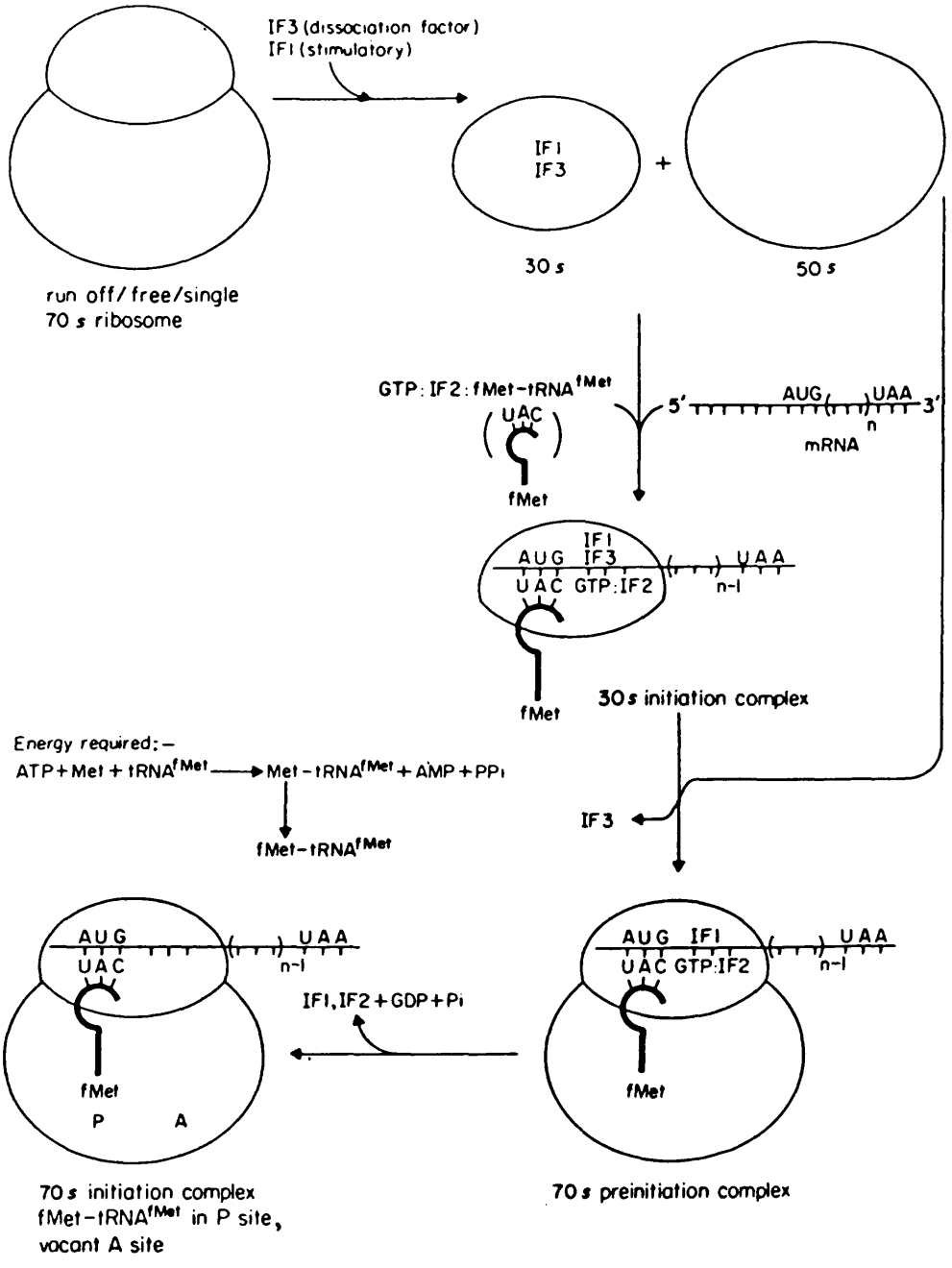


Fig. 1.3. Bacterial protein synthesis. Stage I, Initiation.

: from Mainwaring, W.I.P., et al. (1982) in "Nucleic Acid Biochemistry and Molecular Biology" Blackwell Scientific Publications, Oxford.

own signals for initiation and termination of polypeptide chain synthesis. Each of the genes initiates protein synthesis at a different rate. However, it is difficult to study since in the translation of most bacterial mRNA, the degradation of the 5' end of mRNA can begin while the 3' end of mRNA is still being synthesized (Forchhammer et al., 1972). The segment of a mRNA encoding different protein can have a different half-life so that the determination at the level of mRNA is difficult.

1.6.1.a. Initiation.

There are a number of reviews for the mechanism and the control of the initiation process (Lodish, 1976; Ravel and Groner 1978; and Hunt,1980). The process of initiation is related to how ribosomes find the initiation site on mRNA, how the process is controlled, how many different components are involved and what each individual component does.

In both prokaryotic and eukaryotic, initiation of synthesis of all polypeptide is mediated by a specific met-tRNA (initiation tRNA). In most bacteria, a formyl residue is added to the amino group of the met after the latter is esterified to fMet-tRNA^{Met} (N- formylmethionyl-tRNA^{Met}). By contrast, in eukaryotic cells, the initiator Met-tRNA^{Met} is not formylated. The next step of initiation proceeds by the addition of initiator tRNA and mRNA to the small ribosomal subunit (30S in bacteria and 40S in eukaryote) followed immediately by the joining of the large (50S or 60S) ribosomal subunit. The diagram of initiation stage in bacteria is shown in Fig.1.3.

In bacteria, binding of the initiator tRNA to ribosome requires the initiation factors; IF-1 and IF-2, and GTP and is

dependent on the addition of mRNA. The IF-1 stabilizes the binding of IF-2 to the 30S initiation complex and is required for IF-2 release from the 70S initiation complex. The IF-1 also enhances the rate of dissociation of 70S ribosomes into their component subunit, thereby cooperating with IF-3 in supplying 30S ribosomal subunits, from the 70S ribosome pool. The IF-2 recognizes and promotes the ribosomal binding of initiator tRNA and exhibits a ribosome-dependent GTPase activity. IF-3 acts as a ribosome dissociation factor in that it binds to free 30S ribosomes and prevents their reassociation with 50S subunit. IF-3 also stabilizes the binding of mRNA to the 30S subunit and this reaction also requires ribosomal protein S_1 (Isono and Isono, 1975). However, the situation in bacteria is not so clear cut because the 30S subunit/initiation tRNA complex is not so stable and mRNA seems to bind to the 30S subunit.

Prokaryotes and eukaryotes have evolved different ways of recognizing their mRNAs. The mechanism underlying these processes is, however, unclear and most of the available information is based on the prokaryotic system (Steitz and Jakes,1975). Clearly not every AUG triplet could function as initiation codon and a few nucleotides on either side of the AUG cannot be used as the primary signal. In bacteria, the possibility for selection of the correct binding site may be determinedboth by the sequences and conformation of the mRNA including a specific sequence of nucleotides near the AUG codon and by the small ribosomal subunit. A segment about 30-40 nucleotides long in mRNA is protected from the action of RNase by ribosome binding and the initiator, AUG, of all bacteriophages is approximately in the middle of this ribosome-protected sequence. There is a polypurine rich stretch of

3-9 nucleotides (---5' AGGAGGU 3'---) on the 5' side of the initiation codon and this is complementary to a pyrimidine sequence at the 3' terminus of E.coli 16S rRNA (Steitz and Jakes, 1975; Shine and Dalgarno 1975) and it participates directly in translational initiation. The 3' end of 16S rRNA is necessary for recognition of initiation site on mRNA. The proteins located around the ribosome are required in the binding of mRNA and initiator tRNA during initiation and for stabilizing the RNA-RNA interaction. Some of these proteins are initiation factors such as IF-2 and IF-3 and some are ribosomal protein such as S_1, S_{12} and interference factor ic. These proteins can produce changes in rRNA or mRNA configuration and affect the stability of the interaction between them. At low concentration , factor 3, or ic is required for translation of all natural mRNA (Van Dieijen et al., 1975; Szer et al., 1975) and it blocks translation of poly U and other polynucleotides when present in excess amount (Miller and Wahba, 1974). All these results are obtained from the studies in cell-free protein synthesis. In 1980, Backendorf et al. proposed that the initial rRNA-mRNA interaction may occur some distance away from the initiation sequence. The efficiency of ribosome binding and the efficiency of initiation results from the correct binding of rRNA to the initiation codon on mRNA and this is helped by a region of sequences complementary between the two RNA species.

The last step in initiation is the formation of the 70S complex. The 30S initiation complex which still carries all these IFs, binds a 50S subunit. This reaction involves RNA-protein, protein-protein, and RNA-RNA interaction, the latter may be due to the formation of base pairs between sequences in the 3' terminal regions of 16S and 23S RNA, and IF-3 could be concerned because it

can be cross-linked to sites near the termini of both RNA species (Grunberg-Manago et al., 1978). The IF-3 is last to leave the "70S pre-initiation complex" with initiator tRNA in the P site and IF-2 in the A site. The conformation of IF-2 is changed which reduces its affinity for the ribosome and it is released from the 70S initiation complex. The release of IF-2 also requires IF-1 and the energy is derived from GTP hydrolysis.

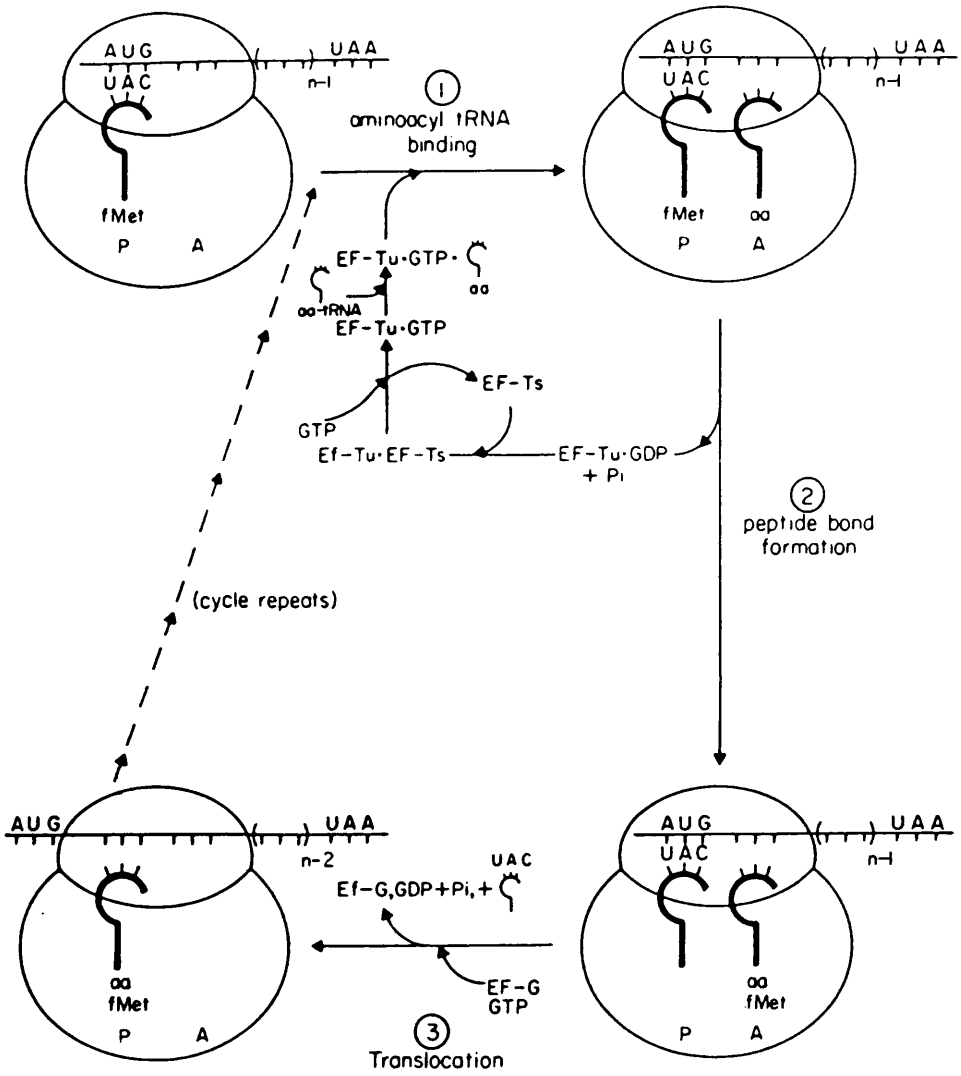
There are several important differences between eukaryotic mRNA and prokaryotic mRNA, including the cap structure at the 5' end and polyA at the 3'end, the nucleotide sequence at the 5' end preceding the initiator codon (leader sequence) which has no uniformity with respect to either length or base sequence and has no coding function, ribosomal protein which is much larger in eukaryotic cells than in bacteria and the number of initiation factors. The process of initiation in eukaryotes is more complicated than in prokaryotic organisms and information has been provided mostly from studies in the rabbit reticulocyte system. This process is briefly described, based on the work of Benne and Hershey (1978).

The first step is the formation of two distinct complexes, one is a binary complex between the small 40S subunit and eIF-3 and the other is ternary complex containing the initiator tRNA, GTP and eIF-2. These two complexes are brought together in the presence of eIF-4C and eIF-3 is required to bind and stabilize the ternary complex to the 40S subunit. In the next step, mRNA is added to the complex and the 40S initiation complex is formed by using the energy from ATP hydrolysis. This stage requires, eIF-1, eIF-4A, eIF-4B and eIF-3, which has a greater effect on mRNA binding than on the binding of $Met\text{-}tRNA_i^{Met}$ (Trachsel et al., 1977), so

that this eIF-3 should be considered as a mRNA binding factor. The work of Kozak (1980 a) indicated that secondary structure of mRNA is not required for mRNA-40S subunit interaction, whereas the fidelity and efficiency of translation does depend on the precise folding, or secondary structure, of mRNA. The eIF-2 is a minor protein of the mRNA particles and it has a high binding affinity for several mRNAs and also for polyA (Shafritz et al., 1976). The final step is the joining of 60S subunit to the 40S initiation complex in the presence of eIF-5, forming the 80S initiation complex. This may occur when the Met-$tRNA_i^{Met}$ anticodon has engaged the initiator, AUG, codon but this is uncertain. The energy for this step is from the hydrolysis of the GTP by the GTPase associated with eFI-5 and thus the initiation stage is complete.

In eukaryotes, the ribosomes always seem to start at the first AUG near to the 5' end in mRNA, in contrast to bacterial ribosomes which can select an internal AUG codon to initiate translation. Both RNA-RNA and protein-RNA interaction may play a role in mRNA recognition and discrimination by the ribosome. At present, the evidence for rRNA-mRNA base pairing (the region near the 3' end of 18S rRNA and the short sequence at 5' side of initiation codon on mRNA) in eukaryotes is weak (Steitz and Jakes, 1975). A cap binding protein (CBP) has now been identified in many eukaryotic cells which specifically aids the formation of a stable initiation complex (Sonenberg et al., 1979); in particular, CBP enhances the interactions between the two initiation factors, eIF-3 and eIF-4B, and mRNA.

Studies by Kozak and Shatkin (1978) and Filipowicz and Haenni (1979) indicated that the 40S subunit binds at the 5'



Energy required per elongation cycle:

$$aa + tRNA + ATP \longrightarrow aa\,tRNA + AMP + PPi$$

$$2GTP \longrightarrow 2GDP + 2Pi$$

Fig. 1.4. Bacterial protein synthesis. Stage II, Elongation.

:from Mainwaring,W.I.P.,et al.(1982) in "Nucleic Acid Biochemistry and Molecular Biology" Blackwell Scientific Publications, Oxford.

terminus of mRNA (leader sequence) and then moves in the 3' direction to the initiation codon. At this time, the 60S subunit joins up with the mRNA-bound 40S subunit and translation is initiated. The molecular basis for recognition between the 40S subunit and the leader sequence is not yet known but migration of the 40S subunit along eukaryotic mRNA supports the "scanning model" which has been proposed by Kozak (1980 b). The 5' cap structure also has an important role in the initiation step of capped mRNA translation as described in section 1.3.2.a.

1.6.1.b. Elongation.

The elongation stage in protein synthesis is defined as the addition of each amino acid to the growing polypeptide chain in a sequence dictated by mRNA (see Clark,1980; Szekely, 1980 for review).There are three steps in the mechanism of the elongation process; (1), the binding of aminoacyl-tRNA to the ribosome; (2), formation of the peptide bond; and (3), translocation. In both prokaryotes and eukaryotes, these three steps are essential but most of the mechaism has been studied in the in vitro system of prokaryotes, especially E.coli.

In the first step of elongation, the elongation factor, (EF-Tu) initially forms a complex with GTP and this complex can bind tightly to any aa-tRNA (except initiator t-RNA). The (aa-tRNA.EF-Tu.GTP) ternary complex interacts with the ribosome at A-site and the anticodon of the aa-tRNA anneals to the corresponding codon on mRNA (Fig 1.4). The conformation of tRNA and 5S rRNA components of the 50S subunit play a role in this binding by forming hydrogen bonds between a specific CGAA sequences of 5S rRNA with the TΨCG sequences of tRNA (Erdmann, 1976). By the

help of a proof reading mechanism and interaction of the ribosomal proteins, either L_{16} which interacts with aa-tRNA and stabilizes in the A site or L_7 and L_{12} which interact with EF-Tu to produce GTPase activity, the translation of the message is carried out with very great precision and the mistakes are estimated at less than 1 per 10^4 codons. GTP hydrolysis is required for the release of the elongation factor in the form of an EF.Tu.GDP complex. Recycling of EF-Tu in an active form occurs with the aid of other elongation factors (EF-Ts) and the use of GTP as energy (Fig. 1.4). No clear data has been obtained about the way in which the EF-Tu.GTP complex binds to aminoacyl-tRNA. The two binary complexes EF-Tu.GDP and EF-Tu.GTP have different conformation. From the study of the three dimensional structure of EF-Tu by Rubin et al. (1981), the GDP-binding site is loaated on the surface of a highly structured part called the tight domain. This evidence may be related to the mechanism of (EF-Tu.GTP.aa-tRNA) ternary complex formation.

Peptide bond formation is the second step of the elongation process and is catalyzed by an enzyme, peptidyl transferase, which is a ribosomal domain on the 50S subunit. Other ribosomal proteins including L_3, L_{11}, L_{16}, L_{18}, L_{20} and a stretch of the 23S RNA are cofactors in this enzymatic reaction (Szekely, 1980). The peptide bond is formed by transferring the carboxyl end of the peptidyl- or fMet- from the CCA terminal to tRNA which bound at the ribosomal P site to the amino group of the aa-tRNA at the A site on ribosome (Fig. 1.4.). This transfer reaction is unique in not requiring energy from the breakdown of either ATP or GTP.

Translocation is the last and most complex step in elongation. The uncharged tRNA is removed from the P site,

peptidyl tRNA is then moved from the A site to the P site and the ribosome moves along the mRNA by one codon in the 5'-3' direction (Fig. 1.4.). The EF-G which combines with protein L_7 and L_{12} and a few other proteins and GTP are required for this step. EF-G does seem to be required for translocation at the normal rate. Following translocation, the GTP is hydrolysed, EF-G leaves the ribosome in the form of EF-G.GDP complex and the next elongation cycle can start. It is not clear how the movement of ribosomes forward by one codon is accomplished, but this may be related to changes in the conformation of mRNA, tRNA, the ribosomal particles themselves and the RNA within the ribosomal particles such as 5S rRNA. EF-Tu and EF-G which have a sequence homology of about 100 aa near the N-terminal of EF-G, bind to the ribosome at a common or overlapping site and their rapid binding and release is essential in the translation system (Orchinnikev et al., 1982).

In eukaryotes, the elongation factors are different from the prokaryotic system and are much less characterized (Grunberg -Manago et al., 1978). For the first step, elongation factors; EF-Tu and EF-Ts; are required in prokaryotes but only one elongation factor ; EF-1; is required in eukaryotes (Clark, 1980). EF-1 is present in the cell both in monomeric form and in the form of aggregated; EF-1_H , heavy form and EF-1_L, light form. GTP may disaggregate these heavy and light forms into monomer which then form the ternary complex (EF-1.GTP.aa-tRNA) (Szekely, 1980). EF-1 has a slightly higher affinity for GTP than for GDP. This probably makes recycling of the factor unnecessary. EF-2 is a soluble factor and is involved in a translocation step as the EF-G in prokaryotes (Clark,1980).

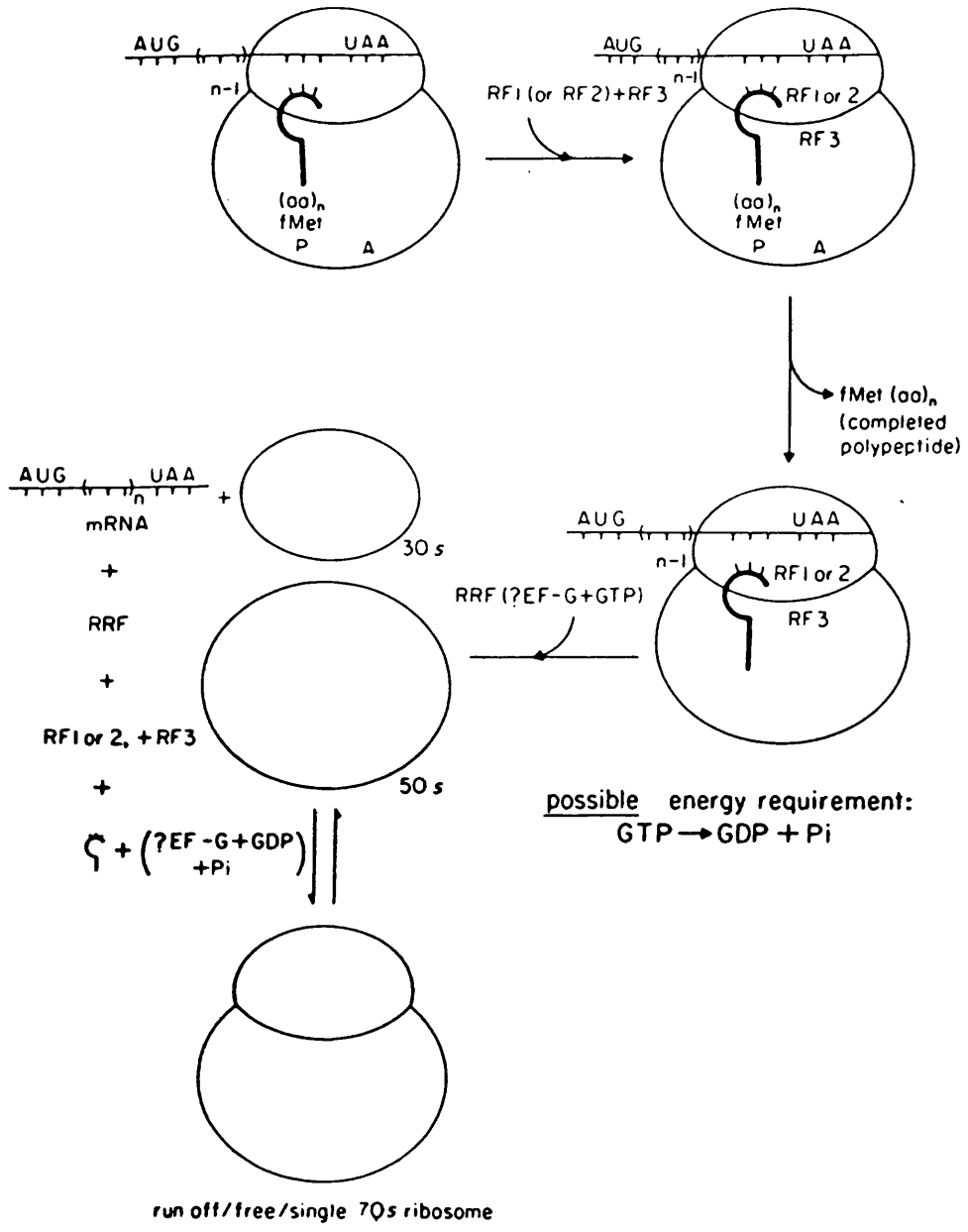


Fig. 1.5. Bacterial protein synthesis. Stage III, Termination.

:from Mainwaring,W.I.P.,et al.(1982) in "Nucleic Acid Biochemistry and Molecular Biology" Blackwell Scientific Publications, Oxford.

1.6.1.c. Termination.

Termination represents the final stage in translation. A mode of peptide chain termination has been obtained from studies in vitro of baterial and mammalian systems. This process differs from chain initiation and elongation since the codon recognition molecules are protein not tRNA and the peptidyl transferase leads to hydrolysis of the peptidyl-tRNA (for review see Caskey, 1980; Grunberg-Manago et al., 1978; Szekely, 1980). The diagram of the termination step in bacteria is shown in Fig.1.5.

Three nonsense codons; UAA, UAG and UGA, serve as termination signals. The actual termination reaction is the hydrolysis of the bond between the tRNA and the carboxyl end of the polypeptide chain and results in the release of the complete polypeptide from ribosomal bound tRNA. This reaction requires release factor and ribosomal peptidyl transferase.

Three release factors are present in E.coli. Two of them are codon specific release factors; RF-1 responds to the termination codons UAA and UAG and RF-2 to UAA and UGA (Scolnick and Caskey, 1969). The third factor, RF-3, promotes binding to and release from ribosomes of RF-1 and RF-2 and interacts with GTP or GDP (Milman et al., 1969). The stimulatory activity of RF-3 is related to ribosomal binding. The eukaryotic release factor is a single protein factor which is nonspecific and can recognize all three termination codons. This release factor interacts with GTP and GDP and promotes an active ribosomal dependent GTPase reaction. By in vitro assay, this release factor requires tetranucleotide (UAAA or UAGA) rather than trinucleotides. However, it appears that the 3' terminal base of these tetranucleotides is not a part of the recognition process but confers an element

of stability on the codon recognition complex.

The release factor binds to the A site (or specific termination codon) of the ribosome and is responsible for codon recognition. The binding and release of this factor is stimulated by GTP and the exact details of this process are not clear. The sequence of the 3' terminus of the 16S rRNA is shown below:

$$3'\ OH\underline{AUU}CCUCC\underline{ACU}A^{5'}--$$

The underlined triplets interact with the termination codon by hydrogen-bonding and this interaction may be involved in the binding of the release factor to the ribosome (Caskey et al., 1977). The ribosomal proteins S_4 and L_4 are involved in peptide chain termination. The protein L_7/L_{12}, S_2 and S_3 are required for release factor binding, while L_{16} and L_{11} affect peptidyl tRNA hydrolysis (Tate et al., 1975). These proteins have been implicated as part of the peptidyl transferase centre and surrounding domain. Release factor is found to cross link to L_2, L_7/L_{12}, L_{11}, S_{17}, S_{21} and S_{18}. There is evidence that the binding domain for the release factor is a region of the interface between the two ribosomal subunits(Brimacombe et al.,1978).Peptidyl transferase has several catalytic activities and it appears most likely that the release factor interacts with the peptidyl transferase to stimulate hydrolysis of the peptidyl-tRNA and release the nascent protein.

1.6.2. The Control of Eukaryotic Cell-free Systems.

In eukaryotes, mRNAs are very stable. They may be translated repeatedly and tend to have a lower rate of protein synthesis than bateria mRNA. It is generally assumed that elongation and termination in all eukaryotic translation occurs at the same rate. Therefore, if translational control mechanisms occur in

eukaryotes, they are most likely to function at the initiation level. Initiation is the rate-limiting step in protein synthesis. Two types of controls might regulate polypeptide chain initiation: Firstly by regulation at the binding of mRNA to eIF-2 and the small 40S subunit, and secondly, the specificity of eukaryotic initiation factor in initiating translation of different mRNAs (Lodish, 1976).

In the eukaryotic initiation step, the formation of the ternary complex, eIF-2.Met-tRNA$_i^{Met}$.GTP,is essential for the attachment of the initiation tRNA to the 40S subunit. A study of the effect of hemin on globin mRNA in reticulocyte shows that hemin affects the eIF-2 kinase by preventing the activation of a cAMP- independent protein kinase by phosphorylation of their subunits(in section 1.6.3.a.) The phosphorylated form of eIF-2 is unable to participate in the usual formation of the ternary complex. In a deficiency of hemin, the translation of globin mRNA is rapidly stopped but the inhibition is reversed by the addition of purified eIF-2. Clearly, the blocking of the function of the initiation factor, eIF-2 by phosphorylation results in control of the polypeptide chain initiation (Ochoa and de Haro, 1979).

The second control is related to message-specific components that would lead to changes in the type of mRNA being translated and thus, in the type of the protein being synthesized. An important test for the specificity of translation of heterologous (foreign) mRNA in different cells was reported by Gurdon et al.(1974) and Woodland et al. (1974). These results show that after microinjection of rabbit globin mRNA into fertilized xenopus eggs, rabbit globin is synthesized in tadpole derived

from these eggs at the same rate as in the egg, and moreover, the developing muscle and nerve cell in this Xenopus laevis were synthesizing rabbit globin as efficiently as erythropoietic cells. These results are supported by Ravel and Groner (1978) who found that the ribosome and initiation factor from one source can translate the mRNA from another source at high efficiency. These results strongly suggested that the translational machinery in all eukaryotic cells has the same specifity towards all mRNA, irrespective of their origin. In contrast to these results, several publications have reported that the homologous mRNA is always translated at better efficiency than heterologous mRNA in cell-free systems and the functional product of one mRNA can inhibit the translation of other mRNAs(Mathews and Osborn, 1974).

Not all of mRNA which is transferred from the nucleus will be translated into protein. Some of them can form a RNP complex which is inactive for translation. There are two forms of globin RNP particles in immature erythrocytes; 15S and 20S (Civelli et al., 1976). Active mRNA can be released from both types of mRNP by deproteinization. The 20S mRNA is a reserved form of globin mRNA and one of the proteins in the 20S mRNP complex prevents translation.

Unfertilized eggs of sea urchin and amphibian also contain mRNP that is not translated. Following fertilization, there is a five-fold or greater rise in protein synthesis and a rise in the proportion of ribosomes engaged in protein synthesis (Gabrielli and Baglioni,1977;Ruderman and Gross, 1975). This increase of mRNA is from the store of mRNP complexes. There is a protein inhibitor which prevents the binding of aminoacyl-tRNA to the cytoplasmic ribosome in the cell of unfertilized eggs

but the mechanism is not known yet (Gambino et al., 1973). Lee-Huang et al. (1977) found that the inhibition in cell sap of dehydrated A.salina cyst is a short RNA, about 20 nucleotides long and rich in U and C. It inhibits aminoacyl.tRNA binding to ribosomes without mRNA specificity, and even blocks translation of synthetic polynucleotides. It is a reasonable hypothesis that inhibition of the mRNA in mRNP complex by protein or short RNA sequences is to prevent their use in protein synthesis.

1.6.3. Inhibition in Translation Systems.

The mechanism of regulation of translation systems is studied by using inhibitors (protein, antibiotics, or chemical reagents) of protein translation in cell-free systems such as rabbit reticulocytes, wheat germ and ascites cell lysate systems. There are two major mechanisms of translation control in eukaryotes. The first is related to the phosphorylation of the initiation factor, eIF-2. Hemin or hemin controlled repressor, oxidized glutathione and double-stranded RNA (ds-RNA)-activated protein kinase are involved in this regulation while the second mechanism inhibits translation by degrading mRNA. This occurs via the action of endonuclease on an oligonucleotides.

The inhibition of protein synthesis by lack of hemin, salt concentration, and addition of dsRNA, low M.W. RNA and oxidized glutathione will be briefly described as follows.

1.6.3.a. Hemin control of translation in reticulocyte.

Protein synthesis in rabbit reticulocytes is controlled by the availability of hemin. In the absence of hemin, which leads to the formation of inhibitor from proinhibitor, protein

synthesis declines rapidly due to a block at the initiation level. These phenomena have been extensively described and reviewed by Ravel and Groner,(1978), Safer and Anderson (1978) and Ochoa and de Haro, (1979).This inhibition has been shown to be related to the formation of inhibitor and this inhibitor is referred to as "the hemin-controlled repressor (HCR)" or the "hemin-controlled inhibitor (HCI)". There are two forms of this inhibitor, the reversible and irreversible forms, dependent on the process of proinhibitor activation which is affected by temperature and N-ethylmaleimide. Reversibly activated HCR, forms by prolonged incubation at 34-37°C for several hr and its formation can be prevented by hemin. From the study of phosphorylase kinase conformation, it has been suggested that activation of HCI might result from a conformational change leading to alteration or release of the catalytic subunit. The irreversible HCR or HCI can be activated by brief incubation (5 min at 34°C) in the presence of N-ethylmaleimide and cannot be inactivated by hemin. HCI is slowly formed and is less active at high potassium concentrations (Suzuki, 1981). This activation mechanism of HCI is still unknown.

Purified HCI appears to have M.W. of 96K on SDS-PAGE and M.W. of about 140K by gel filtration. The HCI is a cyclic AMP-independent protein kinase whose substrate is the smallest subunit (M.W. of 38K) of initiation factor, eIF-2 (Ochoa and de Haro, 1979; Austin and Clemens , 1980;Jagus et al., 1981). Inhibitory effect of HCI on chain initiation in hemin-containing reticulocyte lysates in the presence of ATP seems to be the result of the phosphorylation of eIF-2 and it is not related to the ability of eIF-2 to form ternary or 40S initiation complex. Hemin appears to block the conversion of proinhibitor to inhibitor by blocking the phosphorylation of the 96K

of HCI as well as that of the 38K subunit of eIF-2.

HCI also inhibits binding of Met-tRNA to the 40S ribosomal subunit. This effect requires ATP or GTP hydrolysis and results in reduction of the formation of the ternary complex (Proud and Pain, 1982). Polysome disaggregation is presented in the inhibition due to HCI. This inhibition in reticulocyte lysate, either from the absence of hemin or the addition of HCI in the presence of hemin, can be overcome by the addition of eIF-2. The amount of eIF-2 needed is dependent on the amount of HCI present.

Phosphorylation of eIF-2, 38K protein by HCI can be inhibited by high levels of GTP as well as 3'-5' cAMP, 2 aminopurine (but not hydroxy purine or cAMP), and other purine derivation. GTP appears to block HCI activation possibly by competing with ATP for a site on cAMP-dependent protein kinase. High concentrations of cAMP and 2-aminopurine inhibit protein kinase and nucleotides inhibit HCI action on initiation.

Phosphorylated sugars, including glucose-6-phosphate, 2-deoxyglucose-6-phosphate, fructose-6-phosphate and sedoheptulose 7-phosphate, are known to prevent the effect of hemin-deprivation on translation in reticulocyte lysate and they seem to inhibit HCI formation.

1.6.3.b. Effect of salt-ion on translation in cell-free system.

The efficiency of translation in cell-free systems is dependent on various compounds that are contained in the system, such as ATP, GTP and salts. Weber et al. (1977) have reported that the binding of mRNA to ribosome at the initiation step is inhibited by addition of 150mM KCl. Calcium can enhance the

efficiency of protein synthesis of exogenous mRNA and poly U in reticulocyte lysate when the Mg^{2+} ion concentration is decreased, but theregulatory mechanism is unknown (Ruiz and Krauskopf, 1980; Pantoja et al., 1981). The activity of eIF-2phosphoprotein phosphatase in rabbit reticulocyte is inhibited by a variety of divalent metal ions (Cd^{2+} > Ag^{2+} > Cu^{2+} > Pb^{2+} > Zn^{2+} > Co^{2+} > Sr^{2+} > Mo^{2+}), pyrophosphate, EDTA, NaF, Na HSO_4 and $Na_2S_2O_5$. The inhibition by $Na_2S_2O_5$ is associated with the phosphorylation of eIF-2 and is overcome by addition of exogenous eFI-2 (Ranu and Bhala, 1981).

1.6.3.c. Inhibitory effect of ds-RNA, oxidised glutathione, and low M.W. RNA on translation.

i). Ds-RNA.

Ds- RNA either natural, which can be produced by transcription of DNA or replication of viral RNA genome (such as reovirus, polio or EMC RNA replicative form), or synthetic (a copolymer $(A\text{-}U)_n.(A\text{-}U)_n$ or homopolynucleotide pairs such as $(A)_n.(U)_n$ and $(I)_n.(C)_n$) has been found to be a potent inhibitor of protein synthesis in cell-free systems (Clemens and Vaquero, 1978; Content et al., 1978; Rosen et al, 1981). The mechanism of action of ds-RNA on proteinsynthesis is complex and shows the effect at the level of initiation (including the formation of 80S ribosomal complex and at the elongation step (Rao et al., 1977 a; Sarma et al., 1978).

The inhibition of translation in reticulocyte lysate (in the presence of hemin)by ds-RNA is similar to HCI by activation of the translational inhibitor. This inhibitor, ds-RNA activated inhibitor (DAI), like HCI is a cAMP-independent protein kinase that can cause phosphorylation of the 38K subunit of eIF-2 (Farrell et al., 1977; Lewis et al., 1978) and a decrease in (Met-

$tRNA_f$.40S) ribosomal complex formation. (Kerr et al., 1974). This inhibition is overcome by adding eIF-2 (Clemens et al., 1975). DAI is activated in the presence of ATP and low concentrations of ds-RNA and at high concentrations of ds-RNA, DAI is inactive. In contrast to HCI,ds-RNA induced inhibitor and eIF-2 protein kinase are activated by ds-RNA on the ribosome themselves, without the need for cell sap (Farrell et al., 1977; Levin et al., 1977). Therefore, DAI is ribosome associated and it also activates the phosphorylation of 67K protein. The mechanism of DAI activity is not known yet, it may be a single enzyme or may represent a chain of reactions. Phosphorylation of 67K protein is associated withDAI activation rather than DAI activity (Farrell et al., 1977).

In other.cells such as Krebs ascites cells, the inhibition of mRNA translation occurs only at high concentrations of ds-RNA, about 40 fold higher than the amount neeeded in reticulocyte lysate (Robertson and Mathews, 1973).Grill et al. (1976) have reported that ds-RNA at the low concentration which shows a high inhibitory effect on protein synthesis in reticulocyte lysate has no effect on protein synthesis in wheat germ lysate, even at a high concentration. Inhibition of protein synthesis by ds-RNA may be limited to the mammalian host (Jay et al., 1974) or the initiation factors of wheat germ and reticulocyte may be different since initiation in wheat germ extract is sensitive to temperature (Zehavi-Willner and Postka, 1976).

The toxic effects of ds-RNA are much more potent on protein synthesis in interferon treated cells than in the control cell. Interferon is a glycoprotein which is synthesized by various animal cells after viral infection or by treatment

with inducer (such as ds-RNA). After interferon treatment in the cell-free system, both elongation and initiation steps are inhibited. When the treatment is carried out without ds-RNA, the block appears first in elongation, followed by initiation and then stops (Content et al., 1975). When ds-RNA is added, initiation is blocked earlier (Kerr et al., 1976).

The addition of ds-RNA and ATP tointerferon treated cell extract, or cell extract which was incubated with interferon cell sap, activates a protein kinase(s) that phosphorylated 67K protein and 38K subunit of eIF-2 (as described above). Moreover, addition of ds-RNA and ATP to an extract of interferon treated cells is related to degradation of ss-RNA eg: reovirus mRNA, R_{17}phage RNA [illegible] action is by the formation of a hea[illegible]ch inhibits translation in hemi[illegible]te and mouse L-cell system at su[illegible] et al., 1977). This low M.W. inhi[illegible] oligonucleotide (pppA2'p5'A2'p5'A)[illegible]n, 1978). It appears to have [illegible]tion of either globin or EMC RNA [illegible]t more active to mengo virus RNA than globin mRNA (Farrell et al., 1978). This low M.W. inhibitor probably does not inhibit translation by itself but triggers other reactions, because it has an effect after a lag period of incubation and it inhibits protein synthesis in crude systems and at a low concentration (Hovanessian and Kerr, 1978). Clemens and Williams (1978) have proposed that this low M.W. inhibitor stimulates an endonuclease that degrades mRNA, thus preventing translation, and this proposal is supported by Lewis et al.(1978), Farrell et al. (1978) and Ratner et al.(1978). The low M.W. inhibitor is synthesized from ATP by the enzyme

pppApApApA synthetase. This enzyme is activated by protein kinase which is activated in interferon-treated extract in the presence of ds-RNA and ATP. Two partially purified enzyme fractions, DE1 and DE2, which are isolated from interferon-treated cells, can degrade RNA in the presence of ds-RNA and ATP (Ratner et al., 1978)

ii). Oxidized glutathione (GSSG).

The addition of diamine to a reticulocyte lysate shows an effect on protein synthesis similar to the lack of hemin, since it also induces an inhibitor of phosphorylation of 30K subunit of eIF-2 (Clemens et al., 1975; Levin et al., 1977).

These three compounds; HCI, ds-RNA, and GSSG have a common mechanism of inhibition of translation including decline of protein synthesis starting after a lag period of incubation, disaggregation of polysomes from the mRNA occurring at the time of shut-off, reduction of the number of (Met-$tRNA_f$.40S) ribosomal complexes and the inhibition is reversed by the high concentration of eIF-2 and also by high levels of cAMP and 2-aminopurine.

GSSG-induced inhibition is prevented by a wide range of sugars, such as glucose-6-phosphate, fructose-6-phosphate and 2-deoxy glucose-6-phosphate and several other hexose monophosphate (Wu, 1981) and Giloh et al. (1975) suggested that the level of NADPH may be important.

iii). Low-M.W. RNA.

Oligonucleotides and low-M.W. RNAs have been reported to modulate in vitro protein synthsesis. Oligonucleotides from eukaryotic cell extracts such as chick erythroblast and embryonic chick

muscle, rabbit reticulocyte ribosome and Artemia salina embryo affect translation of mRNA in a non-selective manner (Lee-Huang et al., 1977; Heywood and Kennedy, 1976). Some of these RNA species, the pyrimidine rich inhibitory RNA of Artemia salina embryo, preferentially inhibit chain elongation at the EF-1-dependent binding of aa-tRNA to the 80S ribosome and also inhibit the chain initiation step at a high concentration (Lee-Huang et al., 1977). Other RNA species, a class of low M.W.-uridine-rich dialysable RNA species about 30-40 nucleotides in length and referred to as translational control RNA(tcRNA), from chick embryonic muscle block the initiation step and the inhibition is dependent on its interaction with the 3' polyA tract of mRNA (Heywood and Kennedy, 1976).

The low M.W. RNA as an inhibitory factor of translation in vitro was first isolated from free myosin mRNP particles (Heywood and Kennedy, 1976; Rao et al., 1977 a). Several reports have described the inhibitory effect of low M.W. RNA of nuclear origin (Rao et al.,1977 a; Sarma et al., 1978; Bathurst et al., 1980), however, the physiological significance is still unclear and there are multiple species of inhibitory RNA.

The snRNAs (90-220nucleotides in length) are found in the nuclei of all eukaryotic cells. Little is known about their cellular function. Isolated U1-snRNA inhibits protein synthesis of either liver or Novikoff hepatoma mRNAs in cell-free systems (Rao et al., 1977 a). Another report of snRNA inhibition of cell-free translation included in a less than 10s fraction isolated from guinea pig poly(A)$^+$ nuclear rRNA, which appears to inhibit the translation of milk protein mRNA in several cell-free systems at the level of elongation (Bathurst et al., 1980). This inhibition can be

reversed by increasing the amount of S30 supernatant in wheat germ cell-free system. It is similar to the activity of low M.W. nuclear RNA which was reported by Rao et al. (1977 a) and is in contrast to the low translational activity of another RNA species (Rao et al., 1977 b.). A similar snRNA fraction from lactating rat mammary gland was characterized by Bathurst and Smith (1982). These low M.W. RNAs, which are derived from total poly(A)$^+$ nuclear RNA fraction by sucrose gradient centrifugation, inhibit the translation of several mRNAs but not poly U or poly A and they appear to have different sites of action. There are at least three active inhibitor RNA species, two of them inhibit elongation by interaction with elongation factors while another inhibits initiation of protein synthesis.

There are two types of mRNA-protein complex (mRNP), designated as polysomaland free cytoplasmic RNP particles, found in cytoplasm of eukaryotic cells. Only polysomal mRNAs are active for translation in cell-free systems (Goldenberg and Scherrer, 1981). The inhibition of translation of free cytoplasmic RNP particles may be due to a translational repressor protein (Vincent et al., 1981) or translational control of low M.W. RNA (Lee-Huang et al., 1977; Slegers et al., 1981). The translation of 40S free non-polysomal RNP particles from rat liver in the cell-free system is inhibited by low M.W. RNAs in mRNP. These low M.W. RNAs, of 147, 203, and 263 nucleotides in length associated with the 35S RNP particles that is dissociated from 40S by treatment with EDTA, are powerful inhibitors of protein synthesis and their inhibitory effect may be due to the double-stranded region (Kühn et al., 1982)

Sarma et al. (1978) have reported that a group of low M.W. RNAs (4S to 5.5S RNA, called I-RNA) which are isolated from nuclear

RNA particles of adenovirus-infected HeLa cells, inhibit protein synthesis using mRNA from rabbit reticulocyte and EMV RNA in Ehrlich ascites cell-free systems. The inhibition results from a block at the initiation step by inhibition of the formation of 80S ribosomal complex, and the 5.5 S RNA is thought to be the inhibitor. Mukherjee and Sarkar (1981) have purified a low M.W. RNA species in the 70-90 nucleotides size range (iRNA) from the ribosomal salt wash of chick embryonic muscle and found that the iRNA strongly inhibits the translation of either homologous or heterologous mRNA in nuclease-treated reticulocyte lysate. The inhibition is non-selective and affects the initiation step. It does not appear to be dsRNA, therefore,it is different from other low M.W. inhibitory RNA species including U1 snRNA (Rao et al.,1977 a) and low M.W. RNA in 40S particles (Kühn et al., 1982).

1.7. Systemic Lupus erythematosus (SLE).

SLE is a connective tissue disease that is associated with inflammatory disorders of many tissues and with evidence of abnormal humoral and cellular immune responses. It is a disease of unknown etiology and characterized by multiple clinical manifestations, especially involving the skin,joints and kidneys, and by the presence of autoantibodies, particularly antibody to native DNA. The pathological, clinical and immunological abnormalities in SLE may overlap with other diseases in the connective tissue disease group, which include rheumatoid arthritis (RA), polymyositis and dermatomyositis, progressive systemic sclerosis, Sjogren's syndrome, and MCTD. (Le Roy, 1982). The clinical manifestations, etiology and immunological abnormalities of SLE are described. The autoantibodies which are normally found in the sera of SLE

patients may play an important role in the development of some of the disease manifestations. The characterization of these autoantibodies, including their biological functions, and possible methods for introducing these autoantibodies into cells are also discussed.

1.7.1. General description of SLE.

1.7.1.a. Diagnosis.

A variety of skin manifestations, the involvement of joints, intestinal tract and kidney and the presence of LE cells are first used to characterize the SLE disease. LE cells are detected in over 75% of patients with SLE during active disease (Dubois,1974). Since the correlation of LE cells with disease incidence is not good and LE cells are difficult to detect,this test has been generally replaced by other assays.

The criteria of clinical manifestations (such as arthritis, inflammatory disease, renal disease and Raynaud's syndrome) and biological features (such as antinuclear factor,hypocomplementemia and proteinuria) are now used in the diagnosis of SLE. Criteria of SLE diagnosis have been proposed by the American Rheumatism Association and Dubois, and serve to identify the disease when 4 or more criteria are present (Cohen et al., 1971; Dubois, 1974).

Since many different types of abnormal autoantibodies such as ANA and antibodies to cytoplasmic components have been discovered in SLE patients (Reichlin, 1981; Provost and Reichlin, 1981), it is possible to define SLE on the basis of the presence of certain autoantibodies. The presence of antibodies to native DNA in sera of SLE patients is of major diagnostic significance

and also gives a highly sensitive result. However, this biological characteristic has not been shown to directly cause the disease. AntiDNA antibodies are not exclusive to this disease, some subjects with antiDNA antibodies show no clinical manifestations. In addition, this suggestion is less acceptable now that specific antiDNA antibodies can be measured in other diseases by sensitive techniques such as radioimmunoassay. An intermed--iate approach to the definition of SLE consists of the presence of one or more of the clinical manifestations and the presence of ANA or antibodies to cytoplasmic components at the same time (Tan et al., 1982).

1.7.1.b. Clinical manifestations.

The clinical manifestations of SLE are characterized by involvement of multiple organs. The various clinical manifestations show widely variable incidence and significance in terms of morbidity and mortality.

The symptoms of SLE, together with percentage of frequency are:-
Arthritis and arthralgia (91.6%),
fever (83.6%),
LE cells (75.7%),
skin changes (71.5%),
adenopathy (58.6%),
anemia (56.5%),
anorexia, nausea and vomiting (53.2%),
dysproteinemia (53%),
Myalgia (48.2%),
renal change (46.1%),
Pleuritis (45%),
leukoperia (42.6%),
pericarditis (30.5%),
central nervous system (CNS) (25.5%) (Dubois,1974).

The three most common localizations of diseases are at renal,

cutaneous and articular. Christian (1982) has suggested that SLE may be fatal because of renal failure, CNS disease, cerebral lesion and infection complications. Kidneys and skin are the tissues in SLE patients in which immune complexes deposit and could then result in tissue injury. These tissue injuries are associated with glomerulonephritis, skin lesions, CNS disease, vasculitis, and pericarditis (Koffler et al., 1982).

i). Joints.

Involvement of joints is the most frequent manifestation of SLE (in nearly 92% of Dubois' cases) with evidence of arthritis or both arthritis and arthralgia. The knees are the most commonly involved joint, followed by the wrists and metacapophalangeal joints. Ankles, elbows and shoulders are involved less frequently.

Arthritis occurs particularly in the acute phase, sometimes associated with severe effusion. This disappears rapidly during corticosteroid treatment. Pain may be very intense or mild. In chronic cases, deformation and radiographic abnormalities are very similar to the features of RA and present difficulties in differential diagnosis from arthritis.

Myositis and myalgia usually occur with arthritis. These patients have pain in and between their joints, True polymyositis with evidence of muscle weakness, electromyographic changes typical of polymyositis, vascular myopathy, and necrosis has been reported in untreated SLE patients (Dubois, 1974).

ii). Skin change or dermatological manifestation.

The second most common manifestation of SLE is abnorm-

alities of the skin, hair and mucous membranes. (Dubois, 1974). In acute forms, erythema begins in the face as small red or purple spots and then rapidly extends throughout the face. The rash, which is called the classic 'butterfly' blush is formed. It may or may not be preceded by sun exposure. The second most common erythematous rash seen in SLE patients is a non-specific maculpapular rash which also may occur after sun exposure. It may extend rapidly to the limbs, involving the dorsal part of the hands and feet, forearms, legs and shoulders and the trunk, breast and back.

Immunopathological studies of skin lesions in SLE patients reveal deposits of immunoglobulins (including IgG, IgM, IgA) and complement component (C3) at the dermal-epidermal junction (Schrager and Rothfield, 1976). These patients which showed positive dermal-epidermal fluorescence had more severe disease with high fever and renal disease. However, this finding is not a specific lesion of SLE since the deposition of immunoglobulins and complement component also occurs in SLE patients without skin lesions during active disease (Schrager and Rothfield, 1976). Alopecia is frequent and may appear as patches of hair loss in SLE patients.

iii). Renal disease.

Renal disease is present in 46% of SLE patients (Dubois, 1974) and by renal biopsies with immunofluorescence technique, 75-80% are reported. This technique also showed in nearly all patients a deposition of granular immunoglobulins and complement components on the epithelial surface of the glomerular basement membrane or in the mesangium (Schur; 1975, McCluskey, 1982). Schur (1975) showed that the majority of SLE patients had IgG deposits, more than half had IgM and less than one third had IgA. IgD and

IgE were weakly present and detected infrequently. Complement components, Clq and C_3 were detected in more than half and C_4 was detected infrequently. The kidney may be normal by light microscopy or shows only minimal glomerular change in electron microscopic pattern. The pathological and clinical forms of lupus nephritis in SLE patients can be classified into mild-, severe (diffuse) proliferative-, membranous-and mesangial-lupus nephritis. The immune complexes with C3 are thought to generate chemotatic factors, resulting in the activation of macrophages, phagocytosis of immune complexes and release of lysosomal enzymes (Cochrane and Koffler, 1973). Mannik (1982) has shown that in SLE patients with lupus nephritis, antigen-antibody complexes are deposited in renal glomeruli and subendothelial and mesangial areas but not in subepithelial areas. Affinity of denatured DNA for glomerular basement membrane, which has a net negative charge, has been found and this may explain the deposition of immune complex (Mannik, 1982).

iv). Nervous and blood vessel systems.

SLE is clinically associated with neurological abnormality including CNS disease, convulsive disorders and psychological problems. The CNS disease occurs as two major forms, organic psychosis and seizures (Feinglass et al.,1976). Seizures with hypertension and infection occur in about 15% of SLE patients during active disease while organic brain disease with memory deficits is common in CNS involvement (Feinglass et al., 1976). Severe headaches are associated with either seizures or organic brain disease and disappeared when the patients were treated with corticosteroid. Peripheral neu ropathy, which presents mainly as

sensory and cranial nerve signs, such as facial weakness, ptosis and diplopia, has also occurred in SLE patients (Feinglass et al., 1976).

Immune complexes, (IgG and IgG and IgM but not C3), which mediate vasculitis and are responsible for tissue injury in most organs, are rarely found in the brain of patients with CNS disease. The amount of lymphocytotoxic antibodies in the circulation and IgG antineuronal activity in the CSF has been found to be associated specifically with active CNS disease in SLE patients (Bluestein and Wood.1982). In SLE patients with vasculitis, immune complex deposition is found in the small and large blood vessels the arterioles and venules by immunofluorescence assay. ANAs deposited in blood vessels of the spleen of SLE patients are IgG, IgM, and IgA classes and have, in part, specificity for DNA (Svec and Allen, 1970).

1.7.1.c. Immunological abnormalities.

Both humoral and cell mediated immunity are involved in the immunological abnormalities of lupus disease. These immunological abnormalities include autoantibodies particularly ANAs, immune complexes in tissue and serum manifested as hypocomplementemia and cryoglobulinemia, and abnormalities in cell-mediated immunity.

i). Autoantibodies.

Autoimmunity is a condition in which the organism has an immune response against itself. SLE is characterized by the presence of many different autoantibodies which can be divided into two classes, autoantibodies directed against tissue specific antigens

(thyroid, liver, muscle, adenal) which also include those directed against cellular element of hemopoietic system (lymphocyte,erythrocyte, platelet), and autoantibodies directed against nuclear and cytoplasmic antigens (in section 1.7.2.).

ii). Immune complex-mediated disease.

Immune complex-mediated tissue injury in SLE, which participated by auto antibodies, is clearly associated with glomerulonephritis and with symptoms in other organs such as skin, lung, heart and brain (Koffler et al., 1982). Immune complex deposits in tissue can be detected by immunofluorescence assay. Wilson et al. (1977) showed that the presence of immune complexes is closely related to disease exacerbation in SLE patients and is also associated with cryoglobulinemia, hypocomplementemia and nephritis. Mixed cryoglobulinemia, which involves IgG, IgM, and complement factors (Clq, C3, C4) is commonly found. Winfield et al. (1975) have demonstrated that cryoprecipitates contain both antilymphocyte antibodies (IgM class) and ANAs (to native DNA, ssDNA and RNP).

Activation and fixation of circulating immune complexes in SLE patients are affected by disorders of the complement system. The serum complement level may be affected by varying rates of synthesis and catabolism of complement compounds as well as genetic control of complement levels (Schur, 1975). Serum C4 level is the first to fall in SLE patients. Very low level of Clq showed at active disease state and tended to be associated with high mortality in SLE patients. Low levels of C5 may occur during exacerbation of nephritis and the very low level of C3 is usually accompanied by active and severe nephritis. Complement levels are also depressed in association with extensive skin lesions, arthr-

itis,and hemolytic anemia (Schur, 1975).

iii). Abnormalities of cell-mediated immunity.

Immune reactions are mediated by effector cells; B-lymphocytes, which secrete antibodies, and T lymphocytes, which produce lymphokines and mediate cellular immunity, and phagocytic cells. There are two major types of T lymphocytes, helper and suppressor.

During the active phase of disease in SLE patients, T lymphocytes were reduced while B lymphocytes were normal, decreased or elevated when their number was determined either by formation of rosettes with sheep red cells or by the use of T cell-specific antibodies. The absolute number of suppressor, T lymphocytes was significantly decreased while helper T lymphocytes were slightly reduced (Williams, 1982). It has been demonstrated that cold-reactive lympho-cytotoxic antibodies and immune complexes which interacted with the complement receptors on B lymphocytes, may interfere with the determination of the number of B lymphocytes (De Horatius, 1982). Antibody-dependent lymphocyte mediated cytotoxicity was also decreased in SLE patients especially during active disease. In autopsy studies, SLE patients often show thymic abnormality with many germinal centres containing plasma cells. These thymic abnormalities are not specific for SLE and may be a secondary effect. The mechanism involved in T lymphocyte deficiency in SLE patients is still unclear.

1.7.1.d. Etiology.

The initiation and aggravation of SLE may have a multi-

factorial etiology. The clinical heterogeneity of SLE also shows the involvement of more than one factor. These factors include viral infection, genetic factors, environmental factors and endocrine factors. However, in some cases, the presence of a high level of one factor may cause the disease. Therefore, it is difficult to make definite conclusions regarding actiology.

i). Virus.

Both New Zealand black (NZB) and New Zealand white (NZW) mouse strains, have autoimmune phenomena and disease that resembles human SLE. Type C oncornavirus is involved in its pathogenesis (Yoshiki et al., 1974). Viral glycoprotein with M.W. of 70K is present in a high concentration in the blood and spleen of NZ mice and also is deposited in renal glomeruli as an immune complex (Yoshiki et al., 1974). However, the formation of pathogenic complexes in NZ mice is still unclear. Chronic infection with polyoma virus also enhances the SLE-like expression of NZB disease.

Because of the similarity of SLE to NZ mouse disease, the role of virus, particularly type C virus, has been studied in SLE patients. Levels of serum antibodies directed against RNA viruses such as measles and rubella (Phillips, 1975) and those directed against DNA viruses such as adenovirus and Epstein-Barr viruses (Pincus, 1982), are significantly elevated in SLE patients. This seems to be a reflection of hyperactivity of humoral immunity in SLE rather than any specific viral actiology.

The presence of the inclusions (such as antibodies to type C virus antigens) is not SLE specific because it is also found in lupus nephritis, scleroderma and Sjogren's syndrome

(Winchester, 1978). In addition, antiviral antiserum apparently reacted with only 10% of glomeruli and the reaction was weaker in some positive SLE patients (Winchester, 1978). Subsequently, antibodies eluted from these kidneys are found to have some anti-type C reactivity. These studies suggested type C viruses are involved in the immunopathogenesis of some cases of SLE. However, the role of type C virus in SLE remains a hypothetical (Phillip, 1975) and the results are still inconclusive.

ii). Genetic factors.

SLE affects individuals of all races but varies in different countries and it seems to occur more frequently in females than males. In the United States, black females suffered from SLE more than do white females, while this disease is apparently uncommon in Africa.

Many reports have suggested family clustering of SLE and related rheumatic disease. Therefore, genetic factors must play an important role in SLE. First-degree relatives have been reported to have a higher incidence of ANA (4.33%) than normal controls (0-1%) and also show a high incidence of Raynaud's phenomenon (Block et al., 1975). In many cases, members of the patient's family show only autoimmune manifestations such as hyper gammaglobulinemia and a high frequency of RA. AntiRNA -antibodies, which are related to antilymphocyte antibodies, are found in high levels in the serum of household contacts and relatives of SLE patients (De Horatius et al., 1975). The concordance rated in monozygotic twins is high, including 57% for SLE disease, 71% for ANA incidence and 87% for hypergammaglobulinemia (Block et al., 1975). The rate for this concordance in dizygotic twins appears

to be lower.

iii). Environmental factors.

Sunlight has been found to relate to SLE or to exacerbation of this disease but the incidence of the disease may not relate to the amount of sunlight. Chemicals, such as food additives, which might combine with and alter the structure and function of DNA have also been reported to relate to SLE. In rare cases, the high incidence of ANA lymphocytotoxic antibodies is found in workers who are regularly exposed in laboratories to sera of SLE patients.

Certain drugs may produce the whole or partial clinical or serological feature of lupus-like illness and also induce ANAs. The drugs often involved include procainamide, hydralazine, antiepileptic drugs and anticonvulsants (isoniazid) and other drugs rarely involved, include α-methyldopasulfonamide, penicillinamide and beta-blockers (Hess,1982). Joint pains and swelling, fever, rashes, pericarditis and pulmonary ateleetasis are commonly involved in drug-induced lupus. These clinical symptoms are milder than in patients with spontaneous SLE. Renal lesions and female predominance are also unusual. LE cells and ANA, which is directed against nucleoprotein especially histone or ssDNA, are generally found. There are no antibodies directed against native DNA and against non histone protein antigens. The mechanism for the induction of ANA and lupus by these drugs is obscure. Although in experimental animals, the appearance of ANAs is found with administration of high levels of both isoniazid and hydralazine. The possible mechanisms are release or denaturation of nuclear antigen, drug autoantigenicity after coupling to

serum protein or activation of latent virus. Some inducing drugs (like hydralazine and procainamide) can bind to DNA and then alter its physical and chemical properties.

iv). Endocrine factors.

SLE occurs predominantly in women, which is probably related to the action of female hormones. Talal et al. (1982) have found that androgens improve the disease in female NZB mice by stopping the decrease in interleukin-2 (T cell growth factor), reducing immune complex nephritis and increasing the response of spleen cells to interleukin-2. Castration in male NZB mice induced aggravation of the disease. Oral contraceptives have also been found to lead to development of a lupus-like syndrome. An estrogen (16-α hydroxy oestrone) responsible for endocrine predisposition to SLE is found in some patients with SLE and is also found in clinical liver disease and rheumatoid arthrtis (Lahita et al, 1982).

1.7.2. Autoantibodies against nuclear and cytoplasmic antigen in SLE patients.

SLE is an autoimmune disease of unknown etiology (see Notman et al., 1975; Provost, 1979; Reichlin ,1981). There are two groups of ANAs divided according to their antigen;; those that react with macromolecules (DNA, RNA and nucleohistone) and antibodies to extractable nuclear antigens (Reichlin, 1981). The incidence of these heterogeneous ANAs in sera of SLE patients and other rheumatological diseases is very high. They are considered for diagnosis of the diseases (Farrell and Tan, 1983).

1.7.2.a. Antibodies to macromolecules.

i). Antibodies to DNA.

High titres of antiDNA antibodies are one of the major serological markers of clinical autoimmunity, especially in SLE patients, and occur in 70-75% of SLE patients (Reichlin, 1981). By the indirect immunofluorescence technique, the peripheral nuclear staining pattern is recognized for antiDNA antibodies. The more sensitive radioimmunoassay has demonstrated that these antibodies are also present in normal individuals but the antibody levels are much higher in SLE patients (Notman et al., 1975) and heterogeneity of antiDNA antibodies is found in SLE. A simple, rapid and highly sensitive ELISA procedure has been developed for use in routine, clinical and experimental studies of antiDNA antibodies for determination of sera from SLE patients (Halbert et al., 1981; Pisetsky and Peters, 1981). It has one disadvantage, the difficulty in attaching native DNA to solid phase supports. A high concentration of DNA or an additional binding agent have been used to achieve adequate DNA adherence.

Antibodies to DNA can be divided into three major types. The most prevalent are those that react with sites on both native (or ds)DNA and denatured (or ss)DNA (ds/ssDNA), some react with only dsDNA and some react with only ssDNA (Cohen et al., 1971; Reichlin, 1981; Tan,1982).

Antibodies to ds/ssDNA are present in 50-70% of patients with SLE (Notman et al., 1975). In other rheumatological diseases these antibodies are present in a low level. Antibodies recognize a common antigenic determinant possessed by both ds and ssDNA. A part of the deoxyribose phosphate backbone of DNA is an important component of this antigenic determinant. The class of antibodies

to dsDNA appears to be both IgM and IgG. The IgG antibodies are primarily IgG1 and IgG3. Using monoclonal antibodies to DNA from NZB/NZW and MRL/lpr mice in competitive radioimmunoassay, the antibodies to ds/ssDNA from SLE patients which reacted with the phosphate sugar-backbone also had the ability to cross-react with phospholipids, especially cardiolipin (Koike et al., 1982).

For antibodies specific for dsDNA it has been presumed that the reactive antigenic determinant on DNA should be related to either the double helical conformation or the secondary structure of dsDNA, which consists of deoxyribosephosphate backbone. Great heterogeneity exists in the binding specificity of antibodies to dsDNA in SLE sera and antibodies react with dsDNA fragments varying in size from 20 to 1200 base pair (Papalian et al., 1980). There are two types of antibodies directed against the left-handed Z-DNA helix. The Z DNA helix is organized in a zig-zag pattern, which results in the exposure of bases on its helix as well as phosphate groups (Wang et al., 1981). Casperson and Voss(1983 a, 1983 b) also demonstrated that not only nucleotide bases, the linear DNA backbone and secondary structure but also the nucleotide sequence were important in the recognition of dsDNA by antibodies in SLE sera.

High titres of anti-dsDNA antibodies and immune complexes (antiDNA-dsDNA) in the circulation correlate well with active nephritis in SLE patients (Davis et al., 1977; Cochrane and Koffler, 1973). The initial association rate of DNA to antiDNA shows a closer correlation with disease activity than other factors, such as total antibody concentrations or antibody classes. Francoistron et al. (1982) have developed a new method for detecting DNA-antiDNA complexes in SLE sera using monoclonal anti dsDNA antibodies,

and have found that the presence of this complex is associated with low levels of C4.

Antibodies to ssDNA are found in up to 87% of SLE patients with and without nephritis and are also found in RA, chronic nephritis and procainamide-induced lupus patients (Koffler et al., 1971). Their antigenic determinants are purine and pyrimidine bases(Farrell and Tan, 1983). Many of these antibodies also cross-react extensively with the base determinants of RNA. From the observations that anti ssDNA is present in sera from lupus patients, and because of the appearance of antigen and antibody in cryoprecipitates in circulation, renal lesions and renal eluates, antibodies to ssDNA have been proposed to play a major role in the immunopathogenesis of SLE. The changes in antiDNA antibodies and free serum DNA are closely correlated to clinical manifestations of SLE (Swaak et al., 1982). Alarcon- Segovia et al.(1975) has shown that antibodies in SLE patients are directed to a variety of nucleotides and nucleotide sequences. SsDNA has many potential antigenic determinants. Deven et al.(1978) suggested that antibodies to ssDNA are heterogeneous both within each determinant and between all of the various determinants.

ii). Antibodies to RNA.

Antibodies to ds-and ss-RNA are not diagnostic for SLE but are found more frequently and in high titres in SLE patients (Koffler et al., 1971). Ds-RNA occur in high amounts in the RNA of virally infected tissue, so that the antibodies may represent part of an immune response to a virus.

iii). Antibodies to histone.

Anti histone antibodies are relatively common in SLE. Histones are basic proteins and tend to complex non-specifically with many serum proteins. It is difficult to study immunological reactions with histones by immunodiffusion assay. Tan et al. (1976) have developed a method for detection of histone antibodies by using an immunofluorescence assay with acid-treated tissue. In acid-treated tissue, proteins including histones and non-histones are eluted from the tissue with 0.1N HCl and DNA is not eluted and remains in ds form. By this assay, about 35% of patients with idiopathic SLE possess antibodies to histone while almost all patients (95%) with drug-induced lupus have these antibodies. Antibodies to the individual histones H2A and H2B were detected in patients with drug-induced lupus, while in idiopathic SLE antibodies reacting specifically with histone H1, H2A, H2B, H3, and H4 have been found. More than 50% of the sera from SLE patients had IgM activity to total histones and a slightly lower frequency was observed for IgG antibodies.

1.7.2.b. Antibodies to small nuclear and cytoplasmic ribonucleoproteins.

These soluble antigens are Sm, nRNP, Ro and La and are recognized by antibodies in sera from rheumatic diseases, such as SLE, MCTD, RA, scleroderma, and Sjogren's syndrome (Notman et al., 1975; Provost, 1979; Reichlin, 1981). The presence of these autoantibodies, together with their titre and specificity, is an important clinical feature for diagnosis of the disease. These antigens are complexes of small RNAs, which are not mRNA, tRNA, or rRNA, and protein and are found in the nucleus and cytoplasm of eukaryotic cells (Lenner and Steitz, 1979 and 1981; Zieve, 1981).

Serum from individual SLE patients may contain antibodies of multiple specificities, such as those containing antiSm and antiRNP or antiRo and antiLa.

There are many techniques used to characterize ANA. The most commonly used method is the indirect immunofluorescent technique. The specificity of antibodies cannot be determined by this method. Moreover, as many SLE sera contain antibodies of multiple specificities, distinct patterns of nuclear staining may not be obtained. This method cannot determine the nature of the antigenic molecule.

Analysis of ANAs by Ouchterlony double immunodiffusion in agar or agarose has been a valuable method for defining specificity. Extractable nuclear antigen (ENA), which is prepared from calf thymus or human spleen, is used as a crude source of antigens (Sm, nRNP, Ro and La). The precipitin lines differ antigenically from one another. This method can determine the specificity of antibody but must be carried out with the aid of reference sera containing known precipitin systems. Recently, the pure antigen has been separated by affinity chromatography and used in the more sensitive and specific methods, ELISA and radioimmunoassay (Teppo,1981; White et al., 1981; Venables et al.,1983).

By SDS-PAGE, the proteins are separated, identified and characterized by their relative M.W. (Laemmli, 1970)and a simple efficient and reproducible procedure in transferring protein from gel to nitrocellulose sheets has been developed by Towbin et al. (1979). It is possible to determine the antigenically active proteins to ANAs on nitrocellulose sheetsby detecting the antibody-antigen complexes (White and Hoch,1981;Schrier et al., 1982).

i). Antibodies to Sm.

Antibodies to Sm are found in approximately 25-30% of SLE patients and occur almost exclusively in SLE(Kurata and Tan, 1976). They may serve as a marker antibody for diagnosis of SLE (Notman et al., 1975). Patients with SLE having only this antibody system, are prone to develope renal disease . AntiSm antibodies are primarily of the IgG class (Sabaharval et al., 1983). The presence of antiSm antibodies is usually associated with anti nRNP antibodies. SLE patients with a high incidence of CNS disease (Winfield et al., 1978) and Raynaud's phenomenon (Winn et al., 1979) have antibodies to Sm in these sera. These results were obtained by the positive hemagglutination test with ENA. Therefore, the result may not form a true assay of Sm antibodies only. By using a pure Sm antigen in complement fixation and ELISA, no correlation between antiSm antibodies and CNS, Raynaud's phenomenon and nephritis has been found (Barada et al., 1981). In contrast, Sabaharval et al. (1983) still suggested that antiSm antibodies were related to nephritis, arthritis, serositis, and dermatitis.

The Sm antigen is a non-histone nuclear acidic protein with M.W. about 150K, highly soluble in physiological salt solution. It is insensitive to the action of RNase, DNase, and trypsin except on prolonged exposure. This antigen is largely, but not exclusively,distributed in the nucleus as a speckled staining pattern by immunofluorescence assay. It shows preferential binding to ssDNA over dsDNA (Reyes and Tan,1977). Lerner and Steitz (1979) have reported that this Sm antigen is a complex of snRNAs with 7 polypeptide chains with M.W. between 12K and 35K.

These snRNAs consist of 6 subunits; U1a, U1b, U2, U4, U5 and U6 (Lerner and Steitz, 1979; Lerner et al., 1980; Takano et al., 1980; White et al., 1981; Gibbons et al., 1982; Busch et al., 1982). Some of these proteins are required for antigenicity (Lerner and Steitz, 1979). The Sm determinant is conserved among species from man to insect (Lerner et al., 1980). Lerner et al. (1981a) have confirmed the nuclear localization of Sm snRNP by immunofluorescence using monoclonal antiSm antibodies.

ii). Antibodies to nRNP.

The highest titres and incidence of antibodies to nRNP are present in sera of patients with MCTD (Notman et al., 1975). By the passive hemagglutination technique, these antibodies are also found in lower incidence in SLE, RA and Sjogren's syndrome. In SLE patients, the high incidence of antiRNP antibodies ranges from 23-50% and they often occur with antibodies of other specificities (Kurata and Tan, 1976; Tan et al., 1976). RNP antibodies are characterized by a nuclear speckled immunofluorescence pattern (Provost, 1979). Anti nRNA antibodies are immunoglobulin of the IgG type. Patients with SLE usually have anti nRNP antibodies together with antibodies to Sm while in MCTD only the antibodies to nRNP have been found. The presence of antibodies to nRNP alone in SLE patients shows a low incidence of antibodies to DNA and a low prevalence of renal disease and CNS disease (Notman et al., 1975; Provost, 1979; Sharp, 1982). Nephritis occurs in SLE patients which have anti nRNP antibodies in association with antibodies of other specificities (antiDNA, antiSm, and antiRo) (Reichlin, 1981).

The nRNP antigen is a nuclear non-histone protein with M.W. of 200K and consists of RNA and several protein subunits. It is an unstable antigen and is sensitive to both RNase and trypsin (Takano et al., 1980; Douvas et al., 1979). The snRNAs (U1a and U1b) were recognized by either nRNP antibodies or Sm antibodies (Lerner and Steitz, 1979). The Sm and RNP antigens have independent antigenic determinant (Lerner et al., 1980). The Sm and RNP antigenic determinants are present on discrete small RNP complexes with U1 snRNP having two sites, one for antiSm and one for antiRNP (Lerner et al., 1982). Other reports demonstrated that Sm and RNP represent distinct antigenic sites on the same nuclear complex (Takano et al., 1981; Sharp, 1982). Eilat and Totan (1982) have observed that the autoimmune response in human and murine SLE is restricted to one or a very few antigenic determinants. The nature of the antigenic determinants that are responsible for their activity is still unknown.

The structure of the Sm and RNP antigens and the number of their polypeptide components have been characterized and studied. Variable results have been reported which may be due to differences in types of cells and tissue, method of isolation and detection as well as a real difference in their proteins and snRNAs (MacGillivray et al., 1982).

Lerner and Steitz (1979) have found 7 identical polypeptides (A-G) with M.W. of between 12K and 35K for both Sm and RNP antigen. By affinity chromatography, sucrose gradient centrifugation and SDS-PAGE, only 5 polypeptides with M.W. from 10K to 15K (BDEFG) were associated with RNP whereas the same or similar 5 polypeptides plus 6 additional subunits with M.W. from 21K to 42K associated with Sm antigen only (Gibbonset al., 1982).

Thus RNP and Sm appear to have different numbers of protein components. The identification of 4 polypeptides with M.W. of approximately 13K plus additional polypeptides with M.W. of 30K and 65K are for both Sm and RNP and only two polypeptides of M.W. of 13K, was reported by hemagglutination (Takano et al., 1980). On the other hand, White et al. (1981) have reported that the mixture of RNP and Sm contains 9 polypeptides with M.W. from 9K to 44K and most of these proteins are not actually required for antigenic activity. By sucrose gradient centrifugation, Douvas and Tan (1981) occasionally observed traces of immunological activity in 30-40S hnRNP peak but the preponderance of the antigen is contained in the < 10S fraction. Recently, Hinterberger et al. (1983) and Kinlaw et al. (1983) have shown that U1RNP is composed of a minimum 8 different M.W. polypeptides and 5 of these polypeptides are also found in RNP particles containing U2, U4, U5, and U6.

Major polypeptides of M.W. 30K and 13K (Douvas et al., 1979) and 3 polypeptides of M.W. approximately 65K and 30K (Sharp, 1982) appear to be associated with the nRNP antigenic determinant. Recently, many investigators have found that the nRNP antigenic determinants are related to polypeptides with M.W. of 70K, 40K, and 13K and it has been suggested that the smaller polypeptides are the degraded products (White and Hoch,1981; White et al., 1982; Douvas, 1982; Wieben et al., 1983).

The Sm antigenic determinant appears to be associated with the approximately 13K polypeptide (Takano et al., 1981; White and Hoch,1981; White et al., 1982; Douvas, 1982; Sharp, 1982; Schrier et al., 1982; Buchanan et al., 1983), whereas in other preliminary reports two polypeptides with M.W. of 110K and 28K are

found (Waelti and Hess, 1980).

iii). Antibodies to Ro(SSA).

The SSA antigen is now known to be immunologically identical to the Ro antigen (Alspaugh and Maddison, 1979). Antibodies to Ro or SSA are found in about 30% of SLE patients and 25% of patients with Sjogren's syndrome (Scopelitis et al., 1980). By the counter immunoelectrophoresis technique, these SLE patients with antiRo can be classified into two subgroups; those having antiRo alone and those having both antiRo and antiLa (Wasicek and Reichlin, 1982). Antibodies to Ro rarely occur with antibodies to Sm (Reichlin , 1981). Warsicek and Reichlin (1982) found that patients with SLE which had antibodies to Ro alone, have a high incidence of antibodies to DNA (77%) and serious renal disease (53%). Maddison and Riechlin (1979) have demonstrated that Ro-antiRo immune complexes participate in the development of nephritis. This participation is associated with a decrease of antibodies to Ro in the circulation and enrichment of anti Ro activity in the glomerular eluate from the kidneys. These patients also had anti-ssDNA in their sera and their kidney eluates were also enriched for anti-ssDNA activity. The patients with both antiRo and antiLa (about 75% of Ro patients) had a lower incidence of antibodies to DNA (30%) and a very low incidence of nephritis (9%) (Wasicek and Reichlin,1982) and had milder disease than with antiRo alone.

Maddison et al. (1981) demonstrated that approximately 62% of SLE patients with"ANA-negative SLE" have antiRo precipitin in their sera and 50% of these patients also have antiLa precipitin, while the remainder have anti-ssDNA. The "ANA-negative SLE" class

consists of SLE patients whose sera fail to stain nuclei in immunofluorescence assay.

The Ro antigen is a soluble cytoplasmic acidic glycoprotein with a M.W. of about 100K to 150K which is resistant to both RNase and trypsin. This class of small cytoplasmic RNPs (scRNPs) contains 2 to 5 scRNAs, depending on the mammalian species. RNAs designated hY1 -hY5 are present in Ro scRNPs of human HeLa cells whereas there are two RoRNAs (mY1 and mY2) and 3 RoRNAs (rY1a, rY1b, and rY2) in mouse cells and rat cells, respectively. (Hendrick et al.,1981; Reddy et al., 1983). These RoRNAs range in size from 80-110 nucleotides, possess 5'-triphosphate termini and lack of modified nucleotides(Hendrick et al,1981.)These RoRNAs are different in size and sequence from the U series of snRNAs which are components of the Sm and nRNP antigens (Lerner et al., 1980; Hendrick et al., 1981). These RoRNAs are significantly less abundant than the URNAs and are not so highly conserved from species to species. They are presumed to be part of a RNA-protein complex and their function in cytoplasm is unknown. Ro scRNPs contain the La protein in addition to protein (s) carrying the Ro determinant, therefore, they are also precipitated by AntiLa (Hendrick et al., 1981). The exact number of proteins associated with Ro scRNAs has not been well defined, immunoprecipitation profiles show several high M.W. polypeptides (Francoeur and Mathews , 1982).

iv). Antibodies to La(SSB).

Antibodies to La are found in sera from patients with rheumatic disease, including RA, SLE and Sjogren's syndrome and are strong markers for sicca syndrome (Provost, 1979).

The La antigen is a nuclear RNP molecule which is sensitive

to trypsin and resistant to DNase and RNase (Teppo et al., 1982). Alspaugh and Maddison (1979) have suggested that La antigen is identical to SSB and Ha antigens. Although immunological identity has been shown in the SSB/Ha/La and SSA/Ro system by double diffusion, there are still some differences which have not been resolved. The SSB and Ha antigen were reported as nuclear antigens whereas La antigen was cytoplasmic RNP. Also the SSA antigen was described as a nuclear antigen whereas the Ro antigen was described as a cytoplasmic antigen. Later studies suggested that the La particles were primarily located in the nucleus but they seem to leak out easily during biochemical fractionation of cells (Hendrick et al., 1981; Hardin et al., 1982).

La RNAs are not as highly conserved as the U1RNA. They are significantly less stable than the U or Ro RNAs. Most of the La RNAs are synthesized by RNP polymerase III and possess 5' phosphate with internal modification typical of tRNA, and range from about 80 to 120 nucleotides. The size of the La antigen varies from 30K to 68K, which may be due to the type of cells and extraction procedure used (Francoeur and Mathews, 1982; Teppo et al., 1982). Anti La is also able to precipitate precursor to tRNA and 5S rRNA in human HeLa cells (Rinke and Steitz,1982).

In addition to many cellular La RNAs, at least 4 viral specific RNAs in the form of RNP complexes; Adenovirus encoded RNAs, VAI and VAII and Epstein-Barr-encoded RNA, EBER1 and EBER2 (Lerner et al., 1981a and 1981b;Rosa et al., 1981) are precipitated by antiLa antibodies from SLE patients. These small RNAs: EBER1 and EBER2 are approximately 166 and 172 nucleotides long, respectively, possess 5' pppA terminal and heterogeneity of residues at the 3' terminal with lack of polyA (Lerner et al.,1981;Rosa et al.,1981).

Rosa et al.(1981)demonstrated that host cell protein which binds to viral VA and EBER RNA is required for antigenicity of these viral RNP complexes. The cellular La RNAs are very heterogeneous and their molecular structures have remained obscure.

1.7.3. Introduction of IgG into cells.

The appearance of specific autoantibodies in sera from patients with autoimmune diseases, SLE and MCTD has been reported to be associated with particular clinical signs of disease. Alarcon-Segovia and co-workers, (1978, 1979b) also demonstrated the penetration of antiRNP antibody into live human mononuclear cells (MNC), obtained from these patients, via their Fcγ receptors (Fcγ R). The incidence of intranuclear IgG in MNC of SLE was low and this result may be due to the amount and heterogeneity of their ANAs and the blocking of some Fc receptors (Alarcon-Segovia et al., 1979a). These antiRNP antibodies have also been found to penetrate into T_γ cells via their Fcγ R (Alarcon-Segovia et al., 1979b) resulting in the deletion of their suppressor function. Dysfunction and loss of suppressor cells (T_γ cells) may lead to the self-perpetuation of autoimmune disease, since T cells with Fcγ R seem to behave as suppressor cells in immune regulation and this suppressor function is diminished in diseases where ANA appears (Alarcon-Segovia et al., 1979b). In Fcγ R bearing human T_γ cells, antiRNP antibodies can cause a defect in the progress of activated cells from the G_0+G_1 to S+G2 phase of the cell cycle while anti-native DNA causes activated cells to have an increase in their RNA content without a concomitant increase in their DNA content (Alarcon-Segovia and Llorente, 1983). These reports showed that different effects were caused by different ANAs

upon penetration into living cells. These differences may have pathogenic significance in the disease where these antibodies occur. The effect of anti-RNP on the progression of the cell cycle correlated well with the suggested function of the snRNP (U1) in helping the splicing of mRNA (Lerner et al., 1980).

Antibody penetration into living cells seems to constitute a new mechanism of immunologically mediated damage that may be operative in diseases with autoantibodies, particularly SLE and MCTD. A possible mechanism or effect of ANAs in these CTD may be studied by finding the function of human MNC after introducing high amounts of ANAs into them. There are many procedures for introducing macromolecules into living cells, such as fusion with liposomes or red cell ghosts, or by using cells with abundant Fcγ R (as described above).

1.7.3.a. Red cell-mediated microinjection of macromolecules into mammalian cells.

During hypotonic haemolysis, the swollen red cells can take upexogeneous macromolecules and release the haemoglobin (Schlegel and Rechsteiner, 1978). This lysis can then be reversed by the addition of enough concentrated saline to bring the suspension to physiological salt concentrations. These cells are called gray ghosts (Rechsteiner, 1975). Many macromolecules such as ferritin, myoglobin, IgG, E.coli β -galactosidase, tRNA and mRNA have been shown to be taken up by red cells during their hypotonic haemolysis and passive diffusion is suggested to be the mechanism of uptake (Rechsteiner, 1975; Schlegel and Rechsteiner, 1978; Antman and Livingston, 1980; Boogaard and Dixon, 1983 a). Fusogens such as polyethylene glycol (PEG) or Sendai virus were

used to fuse red cell ghosts with cultured cells (Wasserman et al., 1976; Furusawa, 1980) but fusion efficiencies are generally low and selectivity is lacking. It is also limited by the type of cells that can be treated. PEG mediated microinjection can be enhanced by agglutinating vesicles to cells with phytohemagglutinin (Szoka et al., 1981) or by using vesicle targeting with PEG (Godfrey et al., 1983). The entrapped macromolecules (tRNA, mRNA or immune IgG) have showed their biological activity after transfer into cultured cells (Capecchi et al., 1977; Boogaard and Dixon, 1983 b; Antman and Livingston, 1980). Therefore, red cell microinjection of macromolecules into cell lines is a simple and efficient method and has permitted the introduction of functional macromolecules.

1.7.3.b. Liposomes.

Liposomes have now been used successfully as carriers to introduce a wide range of biologically active molecules and macromolecules into cells both in vivo and in vitro. Liposomes have many advantages, including lack of cytotoxicity, lack of immunogenicity, efficient incorporation of macromolecules into the liposomes under conditions that do not inactivate the incorporated materials and efficiency in transferring entrapped materials into cell cytoplasm. Many substances such as antibodies IgG, horse radish peroxidase, actinomycin D, synthetic ds-RNA and haemoglobin mRNA have been found to be efficiently transferred from liposomes into cells. The interaction of liposomes with cultured cells is a complex phenomenon and could involve these mechanisms; fusion, endocytosis and transfer of phospholipid between liposomes and cell membrane. The actual processes involved remain unclear.

It is related to properties of liposomes such as size, surface charge and lipid composition and optimum conditions with different cell types are achieved by varying the characteristics of the liposomes (for review see Poste and Papahadjopoulos, 1978; Pagano and Weinstein, 1978; Ryman and Tyrrell, 1980; Poste, 1980).

1.8. Aims of this study.

The biological function of the snRNP complexes which are recognized by antiRNP-,antiSm-, antiRo-and antiLa-antibodies has not been well resolved. Some proposed functions of these snRNP complexes, especially U1 RNP, include processing of hnRNP or RNA splicing and regulation of transcription and translation (as described in section 1.4.1,1.5.2,and 1.6.3.c).

Some direct evidence had been obtained to support the proposal that U1 RNA is essential for RNA splicing. In these experiments, antiRNP-, antiSm-, antiRo-and antiLa-antibodies were added directly into permeabilized nuclei from Ad infected cells (Yang et al., 1981) or into whole cell extract of HeLa cells (Padgett et al., 1983 b)were introduced into intact cells using liposomes as a carrier (Lenk et al., 1982). They found that only antiRNP- and antiSm- antibodies specifically inhibit RNA splicing and synthesis of some viral proteins, while antiRo-and antiLa-antibodies have no effect.

To examine the function of these ANAs on both transcription and translation, one possible way is to isolate and purify poly(A)$^+$RNAs from cells which were incubated in the presence or absence of ANAs.These poly(A)$^+$RNAs could then be used as a template to synthesize protein in a cell-free translation system, since the cell-free translation system can be controlled and gives a high efficiency and faithful protein product. An alternative way to determine the effect of ANAs on translation is to add ANAs directly into a cell-free translation system or to introduce them into intact cells before the pulse labelling protein.

Electrophoretic analysis is required to identify the synthesized proteins in anlysis of the effect of these ANAs on transcription and translation.

Chapter Two General Materials and Methods.

2.1. Materials

2.1.1. Biological materials and cell culture supplements.

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2.2. Methods

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2.2.7. Polyacrylamide gel electrophoresis.

2.2.7.a. Single-dimensional separation of synthesized protein.

2.2.7.b. Two-dimensional separation of synthesized protein.

2 General Materials and Methods.

2.1 Materials.

2.1.1. Biological materials and cell culture supplements.

Daudi (human), K 562 (human), RPMI 1788 (human), RPMI 8226 (human), HMy 2 (human), NS 1 (mouse) and X63 (mouse) were cell lines available at the Biochemistry Department (Dr. D.W.Hough).

The sera of SLE patients were kindly supplied by Dr. P. Maddison, Royal National Hospital for Rheumatic Diseases, Bath. Normal human sera came from volunteers in the laboratory.

Normal rat and normal rabbit peripheral blood were provided by the animal house at this University.

The sterile media and supplements; RPMI 1640 with 2g/l of sodium bicarbonate, L-glutamine (200mM) penicillin-streptomycin (5,000 IU/ml and 500µg/ml, respectively), heat inactivated foetal calf serum (FCS) and new born calf serum, were obtained from Flow Laboratories, Ayrshire, Scotland and Gibco, Glasgow, Scotland.

2.1.2. Enzymes and Immunological Reagents.

Rabbit muscle creatine phosphokinase (activity 132 units/mg), bovine pancrease ribonuclease A (protease free, activity 53 units/mg) and Tritirachium album protease K (activity 15 units/mg) were obtained from Sigma, England.

Anti human IgG (γ-chain specific)alkaline phosphatase conjugate (No A-3150) and anti human polyvalent immunoglobulins peroxidase conjugate (No A-8400) were products from Sigma, England. Rabbit anti human $IgM_{k,\lambda}$, rabbit anti human IgG and anti calf red cell IgG were kindly given by Mr.K.M.Thompson (Biochemistry department, Bath University). Staphylococcus aureus adsorbent (SaC) came from Sigma, England.

2.1.3. Radioactive Materials.

L-phenyl (2,3-^{3}H) alanine (specific activity 28.4 Ci/mmole) as an aqueous solution containing 2% ethanol, L-(4,5-^{3}H) leucine (specific activity 61 Ci/mmol) as an aqueous solution containing 2% ethanol; L-^{35}S methionine (specific activity 1000-1500 Ci/mmol) as an aqueous solution containing 0.1% 2-mercaptoethanol; 5,6-(^{3}H) uridine (specific activity 51 Ci/mmol) in 50% aqueous ethanol and iodo (1-^{14}C) acetamide (specific activity 53mCi/mmole) as a powder were purchased from Radiochemical Centre, Amersham International, Bucks., England. ^{125}I-labelled sheep IgG was a gift from Dr.A.M.T.Jehanli (Biochemistry department, Bath University).

2.1.4. Cell-free Translation Systems.

Wheat germ lysate (as suspension in 20mM HEPES, 120mM KCL, 5mM Mg (OAc) and 1mM DTT, protein concentration of 5.4mg/ml) was from Miles Laboratories Ltd. Nuclease-treated wheat germ extract (in buffer consisting of 20mM HEPES, 5mM Mg(OAc), 100mM KCl and 5mM β-mercaptoethanol, 80 A_{260} units/ml) and protein biosynthesis reaction mixture (contains 200mM HEPES, 300mM K(OAc), 1mM Mg(OAc), 2mM ATP, 1mMGTP, 55mM creatine phosphate, 2mg/ml creatine kinase, 800μM spermidine phosphate and 500μM each of 19 amino acids except methionine) were obtained from BRL Ltd., England.

Rabbit reticulocyte lysate as nuclease treated and message dependent (N90) (containing 110-120 mM K(OAc), 1.5-2.5mM Mg(OAc) and 12-620μmolar of each of 20 amino acids) was a product of Amersham International, England.

TMV RNA, rabbit globin mRNA and poly u(5', M.W > 100,000) were purchased from Amersham International, BRL Ltd., and Sigma respectively.

2.1.5. Chromatography Media.

Protein A-sepharose CL-4B (2mg/ml) and Sephadex G-25 were obtained from Pharmacia Fine Chemicals, Sweden, Oligo (dT) cellulose came from Sigma and BRL Ltd. and the poly A binding capacity was 23.3 and 53.5 A_{260} units/g, respectively.

2.1.6. Gel Electrophoresis Media.

Acrylamide and N,N' methylene bisacrylamide were obtained from BDH chemicals. Agarose was from Miles Laboratories Ltd., Nitrocellulose sheet (BA 83) with a pore size of 0.2μm was a product from Schleicher and Schüll, West Germany. Medical X-ray film (Kodak X-Omat) was from Kodak (Britain) Ltd.

Coomassie brilliant blue R250, acridine orange, amido black 10B (naphthol blue black) and 4-chloro-1-naphthol were purchased from Sigma. DMSO and PPO were obtained from BDH chemicals and Sigma, respectively.

Standard protein markers were β-galactosidase (M.W. 116K), catalase (M.W. 62K), lactate dehydrogenase (M.W. 36K), myoglobin (M.W. 17.5K), myosin (200K), BSA (M.W. 68K), cytochrome c(M.W. 12K), trypsin inhibitor (M.W. 21.5K), RNase (M.W. 13.7K), Ig light chain (25K) and Ig heavy chain (γ,50K; μ, 73K).

2.1.7. Chemicals.

Iodoacetamide, negrosin, nonidet P-40 and folin and ciocalteu's phenol reagent were from BDH chemicals. Spermine tetrahydrochloride, DTT, p-nitrophenylphosphate, cycloheximide, aurin tricarboxylic acid, and diethylpyrocarbonate came from Sigma. Guanidine thiocyanate was a product of Fluka AG, chemische, Fabrik CH-9470 Buchs. Polyethylene glycol 4000 was from Merck.

Phosphatidyl choline (from frozen egg yolk) and cholesterol were obtained from Sigma. Trypan blue as a 0.5% solution in saline and Ficoll-Paque came from Flow Laboratories.

All other chemicals were of analytical grade and came from BDH chemicals and Sigma.

2.1.8. Buffers and Solutions.

The buffers and solutions were prepared with glass distilled or double distilled water. Phosphate buffer salts Dulbecco's formula without magnesium and calcium came as a tablet from Flow Laboratories. Other buffers such as citrate phosphate buffer,pH3, 0.1M; phosphate buffer saline (PBS), pH7.2, 0.15M and phosphate buffer pH6.5,0.5M were made as described in Hudson and Hay (1980).

The scintillation fluid consisted of 5g of PPO, 700ml of toluene and 300ml of triton X-100.

2.1.9. Instruments.

Homogenizer was from MSE and B.Bram Melsungen. Beckman ultracentrifuge model L5-65 and model L5-50B were used for maximum speed of 65,000 rpm and 50,000 rpm, respectively. Bench centrifuge and micro-centaur from MSE were also used. Ultraviolet spectrophotometer, SP 8-100 UV from Pye Unicam and CE 212-variable wavelength UV monitor from Cecil Instruments were used with recorder. The absorbance spectra from gel and X-ray film were scanned using a Unicam SP1800 UV spectrophotometer from Pye Unicam. Manual reader from Gilford was used to measure absorbance at 405 nm for ELISA. Liquid scintillation spectrophotometer, model Tri-carb was from Packard and gamma counter, model 128 Ultrogamma was from LKB, Wallac. Light microscope(Zeiss) with UV light and Leitz Orthroplan microscope with UV light were used in fluorescence experiments.

Trans-blot cell was from Bio-Rad laboratories.

2.2. General Methods.

2.2.1. Cellular Methods.

2.2.1.a. Cell Culture.

Cell lines were cultured continuously in RPMI 1640 medium with 10-20% of heat-activated foetal calf serum or newborn calf serum, 2g/l of sodium bicarbonate, 200IU of penicillin and 200µg of streptomycin at 37°C in an incubator with 5% CO_2 in air. The cell concentration usually reaches 1 to 1.5 X 10^6 cells/ml within 3-4 days. The cells were then subcultured by dilution with fresh medium to 200,000 - 500,000 cells/ml.

2.2.1.b. Cell Viability.

Cell viability was determined using a solution of either trypan blue or negrosin. The cell suspension was mixed with 0.2% trypan blue or negrosin in saline at a ratio of 1:2. The cells were counted within 3 to 5 min for trypan blue and after 5 min for negrosin, with 40X objective, using hemocytometer. The dead cells absorbed dye and the percentage viability was given by the equation:-

$$\% \text{ viable cells} = \frac{\text{Total number of cells - number of dead cells}}{\text{Total number of cells}} \times 100$$

2.2.1.c. Preparation of Peripheral Blood Lymphocytes.

Heparinized blood (20 IU/ml) was diluted with an equal volume of saline and layered on to Ficoll-paque. After centrifugation at 400g for 30 min at room temperature, the lymphocytes were removed from the interface layer between plasma and Ficoll-paque. The cells were washed twice with PBS and used for the extraction of RNA.

2.2.1d. Determination of Fc Receptor on Cell Surface by Rosette Assay.

Formation of calf red cell and anti calf red cell complexes.

Calf red cells were washed three times with ice-cold PBS and resuspended in PBS at a concentration of 1% or 2% (V/V). The required amount of anti calf red cell IgG to be used to react with calf red cells was determined by haemagglutination test. A concentration of antibody which just caused agglutination was used. The anti calf red cell IgG was serially diluted in 25µl of PBS in a microtiter tray. 25µl of 1% calf red cells in PBS was then added to each well, mixed and left at 37°C for 30 min before reading the end point.

To prepare IgG-coated calf RBC, the appropriate amount (1/8 dilution) of anti calf red cells IgG was added to 1ml of 2% (V/V) of washed calf red cells, dropwised while gently mixing on a vortex mixer and further mixed for 1 min. The mixture was incubated at 37°C for 30 min, washed twice with PBS and resuspended in 1ml of PBS containing 2% (W/V) of BSA.

Rosette Counting.

About 1 X 10^6 cells (target cells) were washed twice with PBS, and resuspended in 0.2ml of PBS. 100µl of this cell suspension was mixed with 200µl (2% (V/V)) of calf red cell-anti calf red cell IgG complexes and incubated at room temperature for 60 min. One drop of 1% toluidine blue was added and the cells were gently resuspended by hand inversion and left at room temperature for 30-60 min. The cells were resuspended as before and counted under a light microscope. The cells with more than 2 red cells attached were taken as rosettes and 200 cells were counted. The percentage of rosette-forming cells was determined.

Rosettes with 2-4 bounded red cells were considered to be 'weak rosettes' while those with more than 4 red cells as 'strong rosettes'.

2.2.1.e. Cell Lines.

Daudi cell was a lymphoid cell derived from the Burkitt lymphoma. It expressed a 7-S δ MIg both within cells and on their external surface membrane (Marchalonis et al.,1974) and it does not contain Fc receptor or secrete IgM (Eilliot and Takacs,1979).

K562 cell line was originally established by Lozzio and Lozzio (1975) from the pleural effusion of a patient with chronic myelogenous leukaemia in terminal blast crisis and was thought to be an immature myeloid cell line and to lack Ig, EBV genome and EBV receptor. It was not considered as a B-cell line but had some T cell properties (Klein et al.,1976). It is strongly positive for Ig Fc receptors and pinocytosis but does not phagocytose or mediate ab-dependent phagocytosis or cytolysis (Klein et al.,1976). Later it was established as an immature erythroid cell line (Gahmberg and Andersson,1981). It synthesized glycophorin A, which is a major sialoglycoprotein on human erythrocytes (Andersson et al.,1979), and also synthesized ferritin (Rutherford et al., 1981). It can be induced to differentiate and synthesize haemoglobin by hemin (Rutherford et al.,1981).

RPMI1788 cell is a human lymphoblastoid cell line which produces and secretes IgM (λ) in varying proportions of monomeric and pentameric forms. It produced a 2-fold molar excess of light(λ) chains over heavy chains (μ), but did not secrete the excess light chains. It contains a minor μ-chain-encoding mRNA, which sediments at 19S and codes for a 67,400 dalton polypeptide, in addition

to the major μ-chain-encoding mRNA, which sediments at 18S and codes for a 65,000 dalton polypeptide(Molgaard et al.,1981).

RPMI8226 cell line derived from a patient with multiple myeloma. It actively produced only λ-type light chains of Ig which were secreted into the medium (Matsuokay et al.,1967).

NS1 cell line derived from MOPC-21, a BALB/C mouse myeloma cell lines. It is unable to produce a heavy chain of Ig. NS 1-Ag 4-1, which synthesized but did not secrete k type light chain, was used (Hudson and Hay,1980).

X63 mouse cell line has been developed for somatic cell hybridization with immune spleen cells to generate antibody-producing hybrid cell lines (Kearney et al.,1979). It did not synthesize or secrete either light or heavy chains and hybrid cells derived with this parental cell line will only produce antibody of the spleen cell parent.

HMy2 cell line is a rapidly-growing and HAT sensitive human cell line. It has been derived from the ARH-77 human plasma cell leukaemia and produced IgG, (k) (Burk et al.,1978). It is Epstein-Barr nuclear antigen positive and is capable of reproducibly giving rise to stable hybrids when fused with human lymphocytes from a variety of sources.(Edward et al.,1982).

2.2.2. Sterile Techniques.

All glassware and plasticware that is used in cell culture and preparation of RNA should be sterilized by autoclaving. Glassware was baked at 200°-300°C for at least 6 hr or autoclaved. Plasticware was treated with 0.2% diethylpyrocarbonate for 30 min, washed thoroughly with distilled water and then autoclaved for 45 min.

The solutions and buffers were prepared with double-distilled water and sterilized by autoclaving or by filtration through a 0.20μm sterile filter (microflow 25 from Flow Laboratory). The sucrose solution was treated with diethylpyrocarbonate. 50μl of diethylpyrocarbonate was added to 100ml of solution, shaken at room temperature and placed in a bath of boiling water for 15-30min. The solution was shaken vigorously many times while still hot to remove CO_2 and ethanol. This is similar to the method used by Palmiter (1974).

2.2.3. Preparation of Human IgG.

By using protein A-sepharose CL-4B affinity chromatography.

The affinity column (Protein A-sepharose CL-4B about 5ml volume) was initially washed with 0.1M citrate phosphate buffer pH3 and then equilibrated with PBS. 1 to 5ml of sera from normal individuals or SLE patients were applied to the equilibrated column and unbound protein was washed through with PBS until no more protein left the column ($A_{280} < 0.01$). The bound protein (IgG) was then eluted with 0.1M citrate phosphate buffer pH3. This fraction was collected on ice and immediately dialysed against PBS at 4°C, overnight. This purified IgG fraction was concentrated with a Millipore Minicon ultrafiltration unit to a concentration of 4-10mg/ml, aliquoted and stored at -20°C.

By ammonium sulphate precipitation. (Johnston and Thorpe (1982)).

Na_2SO_4 (3.4g) was added to human serum (10ml) at room temperature. The mixture was stirred to dissolve Na_2SO_4 (3.4g) and incubated at 25°C for 30 min. The precipitate (about 1.5ml) was collected by centrifugation at 3000g for 30 min at 25°C and redissolved in water (5ml). This solution was warmed to 25°C and 0.4g of Na_2SO_4 was then added to make 14% W/V. The mixture was stirred to dissolve Na_2SO_4 and incubated at 25°C for 30 min. The precipitate was collected and redissolved in water (3ml). This IgG solution was dialysed against cold PBS overnight, aliquoted and stored at -20°C.

2.2.4. Estimation of Protein Concentration.

By absorbance measurement.

The absorbance of the protein solution was determined at the wavelength of 280nm. The concentration of IgG solution was calculated from the extinction coefficient value, $280E^{1\%}_{cm} = 13.5$.

By the Lowry method (1951).

Five to 20µl of protein solution was made up to 100µl with distilled water in a cuvette, mixed with 100µl of reaction mixture containing 0.02% potassium sodium tartate and 0.01% of copper sulphate and left for 10 min at room temperature. 100µl of Folin-Ciocalteu's phenol reagent (diluted with distilled water at a ratio of 1:2) was added and incubated at room temperature for 30 min before reading the absorbance at wavelength of 750nm. The concentration of protein was determined from a calibration curve prepared with BSA as standard.

2.2.5. Determination of Specificity of ANAs.

By double immunodiffusion (Ouchterlony, 1958).

Five ml of 0.6% agarose in PBS was melted and poured into a 5cm petri dish to give a depth of 3-5mm. The wells of 3mm or5mm in diameter, were made with a cork borer and filled with 40µl of antigen (human spleen extract or calf thymus extract) in the centre well and 20µl of antibodies (serum or IgG solution) in the outer wells. The petri dish was kept in a moist chamber and incubated at room temperature, overnight.

By ELISA for IgG antibodies to Ro, La and DNA.

The well of a Gilford micro ELISA cuvette was coated with 0.25ml of antigen solution (see note 1) and incubated for one hr at 37°C and then overnight at 4°C. The wells were emptied by suction, incubated with 0.25ml of 1% (W/V) BSA in PBS-tween (0.05% V/V) at 37°C for 20 min and then washed three times with PBS-tween for a total of 60 min. 0.25ml of IgG or serum dilution (see note 2) was added and incubated overnight at 4°C; this was followed by washing three times with PBS-tween for a total of 60 min. After incubation of the antigen-antibody complex with 0.25ml of anti-human IgG (γ-chain specific) alkaline phosphatase conjugate (1:1000 dilution in PBS-tween) for two hr at 37°C, the wells were washed three times with PBS-tween for a total of 60 min and 0.2ml of p-nitrophenylphosphate (1mg/ml in 0.05M Na_2CO_3-HCO_3 buffer pH9.8 containing 1mM $MgCl_2$) was added and incubated at room temperature until A_{405} in the maximum well reached 2 absorbance units. The reaction was then stopped in all wells with 0.05ml of 1N NaOH before reading the absorbance at 405nm using a Gilford manual reader.

Note 1). For anti DNA antibodies, calf thymus DNA at a concentration of 1µg/ml in saline sodium citrate buffer pH8 was used as an antigen. Antigen Ro purified from human spleen extract at concentration of 10µg/ml in PBS was used for anti Ro antibodies while antigen La purified from calf thymus extract was used with anti La antibodies at concentration 1µg/ ml in PBS.

Note 2). IgG solution of anti DNA antibodies was diluted with PBS-tween containing 1% BSAto a dilution value of 1/10,1/50 and 1/250. For anti Ro and anti La antibodies, IgG solution was diluted in PBS-tween containing 1% BSA at dilution 1/100, 1/200, and 1/500, while serum was used at a dilution of 1/50, 1/250, and 1/500.

2.2.6. Protein Synthesis in Cell-free Translation System and Determination of Radioactivity.

Translation of poly u and poly(A)$^+$RNA from tissues and cell lines in wheat germ lysate cell-free system.

The composition of 25µl reaction mixture is shown below:-

Component	Final concentration
HEPES pH7.4	28.9mM
DTT	2.75mM
KCl	64mM
Mg(OAc) or Mg(OAc) and spermidine HCl	3.5mM or 2.5mM and 0.2mM
ATP	1mM
GTP	20µM
creatine phosphate	8mM
creatine kinase	1µg
wheat germ lysate*	3.5µl
poly u	20µg
^{3}H-Phe	0.2-1.0µCi

* was a product from Miles Laboratories.

Each of 19 amino acids (except Phe or Leu) at a concentration of 50μM and 0.5-1.0μCi of ^{3}H-Phe or 0.5-2μCi of ^{3}H-Leu were used in translation of poly(A)$^{+}$RNA or total RNA from tissues and cell lines.

The reaction mixture was incubated at 25°C for 90 min and 10μl of reaction mixture was taken and added to 2ml of ice cold 20% TCA containing Phe (2mg/ml) and left on ice for at least one hr. Precipitate was collected under vacuum on GF/C filter (Whatman) presoaked in 20% TCA and washed with 15ml of 20% TCA containing Phe (2mg/ml) and then with 10ml of 95% ethanol. The filter was dried, placed into 3ml of scintillation fluid and radioactivity was counted using liquid scintillation spectrophotometer. This method was an adaptation of the procedure of Robert and Paterson (1973) and Marcu and Dudock (1974).

Translation of TMV RNA in wheat germ lysate cell-free system.

Wheat germ lysate and reaction mixture were obtained from BRL Ltd, and their compositions are shown in the Materials section 2.1.4 above. Each 15μl reaction mixture contained 5μl of wheat germ lysate, 1.5μl of reaction mixture, 1μl of 500mM K(OAc),0.5μl of 20mM Mg(OAc), 1-2μl of ^{35}S-Met(about 20μCi) 0.08μl of TMV RNA and 5-6μl of sterile water. The reaction mixture was incubated at 25°C for 120 min.

In order to determine radioactivity, 1μl reaction mixture was spotted on a GF/C glass filter. The dried filters were put into 100ml of 10% TCA and boiled for 10 min. The filters were washed twice with 10% TCA and twice with 95% ethanol allowing 10 min for each wash. The filters were air dried and radioactivity was counted in 3ml of scintillation fluid.

Protein Synthesis in rabbit reticulocyte lysate cell-free system.

The reaction mixture (25µl) consisted of 10 to 20µl (40% to 80% (V/V)) of rabbit reticulocyte lysate, 20-30µCi of ^{35}S-Met or 2-3µCi of ^{3}H-Leu and TMV RNA, globin mRNA or poly(A)$^{+}$RNA at concentration of 0.05µl, 0.5µg or 0.01to 2 A_{260} units, respectively. Incubation was carried out at 30°C for 60 min. To determine radioactivity, 1-3µl of reaction mixture was added to 0.5ml of 1N NaOH containing 5% (V/V) of 30% H_2O_2 and incubated at 37°C for 10 min. The protein was precipitated with 2ml of ice cold 25% TCA containing 2% (W/V) casein hydrolysate and left on ice for at least 30 min. The precipitate was collected by filtration on a Whatman GF/C glass filter that was presoaked with 8%TCA and washed twice with 3ml of ice cold 8% TCA. The precipitate was dried and radioactivity was counted in 3ml of scintillation fluid. The method followed Pelham and Jackson (1976) with some modification.

2.2.7. Polyacrylamide Gel Electrophoresis.

2.2.7.a. Single-dimensional separation of synthesized protein.

The separation was performed according to the method of Laemmli (1970). This system is a discontinuous pH system in which the stacking gel contains 0.12M Tris-HCl pH6.8 and the resolving gel has 0.37M Tris-HCl pH8.8. The electrode buffer was 0.024M Tris-HCl pH8.3, 0.19M glycine and 0.1% SDS.

Preparation of stacking gel and resolving gel.

The cylindrical gel in a glass tube (10cm long and 0.7cm inner diameter) had 7.5% acrylamide with 0.37M Tris-HCl pH8.8, 0.1% SDS, 0.0002% ammonium persulphate and TEMED as resolving gel. The gels were left to set for at least 4 hr. The stacking gel,

3% acrylamide with 0.12M Tris-HCl pH6.8, 0.1% SDS, 0.0002% ammonium persulphate and TEMED, was then added and left for about 30 min before use.

The size of the vertical slab gel was 140mm X 160mm with a thickness of 1-1.5mm. The resolving gel was a gradient gel of 5-15% acrylamide. 15 to 16ml of 5% acrylamide that contained 0.37M Tris-HCl pH8.8, 0.15% SDS, ammonium persulphate and TEMED were mixed and a linear gradient was generated with 15-16ml of 15% acrylamide containing the same mixture as 5% acrylamide. The gel solution was degassed before filling the gradient mixer and transfered into slab gel by using a peristaltic pump at 2ml/min. The gel was left for at least four hr or overnight. Like the cylindrical gel,the stacking gel used was also 3% acrylamide. The sample wells were formed in the stacking gel by using a Teflon comb. The stacking gel was allowed to polymerize for 15-20 min before removing the Teflon comb, and the wells were rinsed with water.

These gels contained acrylamide and bisacrylamide at the ratio of 30/0.8.

Preparation of samples and markers.

The translation product of poly u in the wheat germ cell-free system was precipitated with 20% TCA. The precipitate was collected by centrifugation, washed twice with 95% ethanol, dried and dissolved in 100µl of 0.05M Tris-HCl pH6.8 and 2% SDS, 5% mercaptoethanol, 0.0001% bromophenol blue and a drop of glycerol were added, and the mixture boiled for 5 min.

For the translation product of RNAs in rabbit reticulocyte lysate, 5-10µl of reaction mixture was treated with 10-20µl of

10mM EDTA containing RNase A (50µg/ml) by incubation at 37°C for 15 min and used immediately or kept at -20°C. The RNase-treated sample (15-30µl) was mixed with 5 to 10µl of 10% SDS, 5µl of mercaptoethanol and 5µl of 0.001% bromophenol blue in 50% glycerol, and then heated at 100°C for 5 min before applying to the gel.

Many proteins (described in Materials section 2.1.6.) were used as a standard marker including labelled proteins, ^{14}C-BSA, ^{14}C-trypsin inhibitor and ^{14}C-RNase A. These labelled proteins were prepared according to the method of Hirs (1967). 50µg of protein in 10µl of 0.1M Tris-HCl pH8.5 containing 2mM EDTA and 5M guanidine HCl were incubated with 0.05M DTT at 30°C for 30 min. 0.03M ^{14}C-iodoacetamide was then added. Carboxymethylation was allowed to occur under nitrogen gas and in darkness at 37°C for 2 hr. The labelled proteins were purified by passing them through the Sephadex G-25 column using 0.625M Tris-HCl pH6.8 and 2% SDS as a buffer and were checked by SDS-PAGE (Fig 2.2).

Running of the gel.

The upper and lower chambers of the electrophoresis apparatus were filled with the same electrode buffer and the positive electrode was in the lower chamber. Electrophoresis was performed for about 30-45 min at a constant voltage of 80 volts for stacking. The voltage was increased to 110-130 volts and the time was based on bromophenol blue migration; about 90 min for cylindrical gel and about 6 hr for slab gel.

Detection of separated protein on gel.

By staining with Coomassie blue.

The length of the gel and the distance moved by bromo-

Table 2.1. The average value of log %T for standard protein markers that were used on 5-15% SDS-PAGE.

Standard protein marker	M.W.(K)	Log %T±S.D.	no. of estimation
Myosin	200	0.802±0.012	8
β-galactosidase	116	0.879±0.014	9
BSA	68	0.937±0.019	12
Catalase	62	0.951±0.014	5
Lactate dehydrogenase	36	1.019±0.016	9
Trypsin inhibitor	21.5	1.092±0.017	8
RNase	13.7	1.105±0.018	8
Cytocrome C	12	1.104±0.027	6

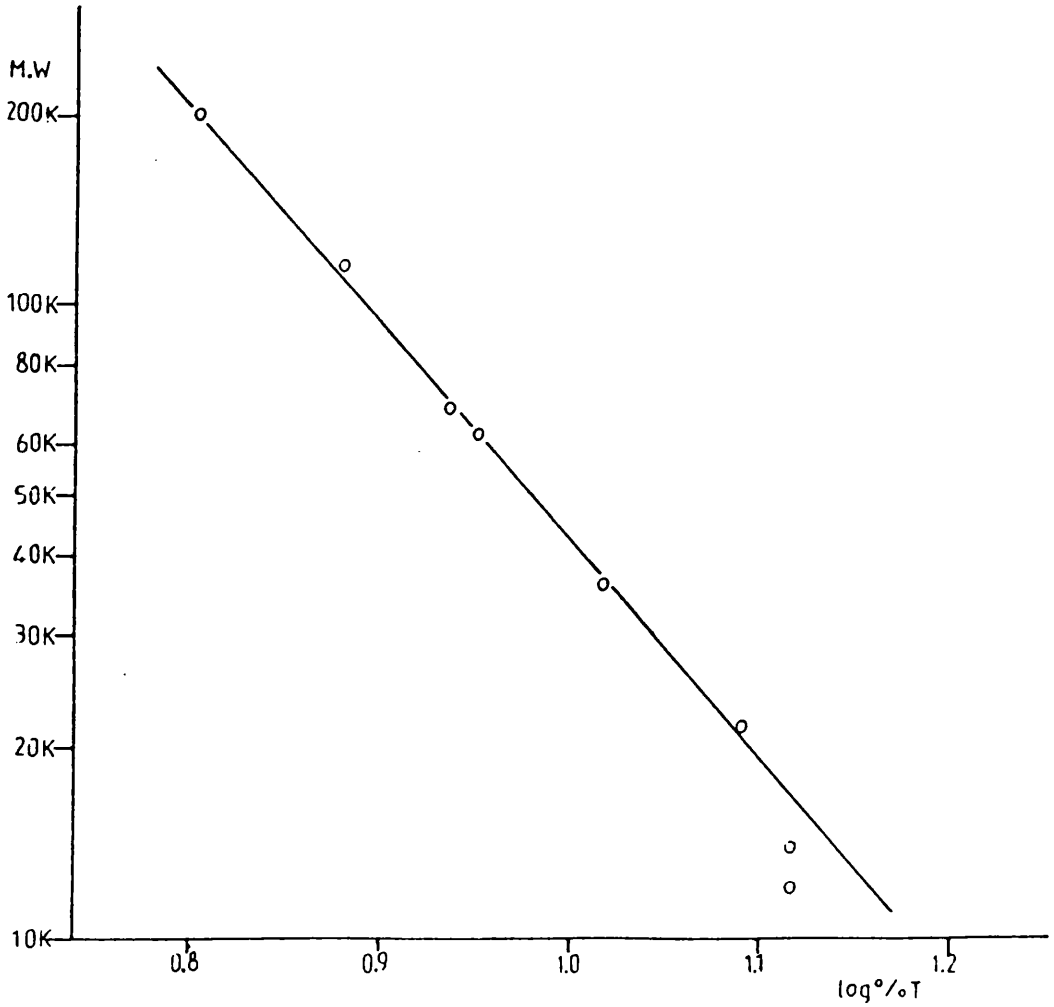


Fig. 2.1. Calibration curve of $\log_{10}$ protein M.W. versus log %T for 5-15% linear gradient SDS-PAGE..

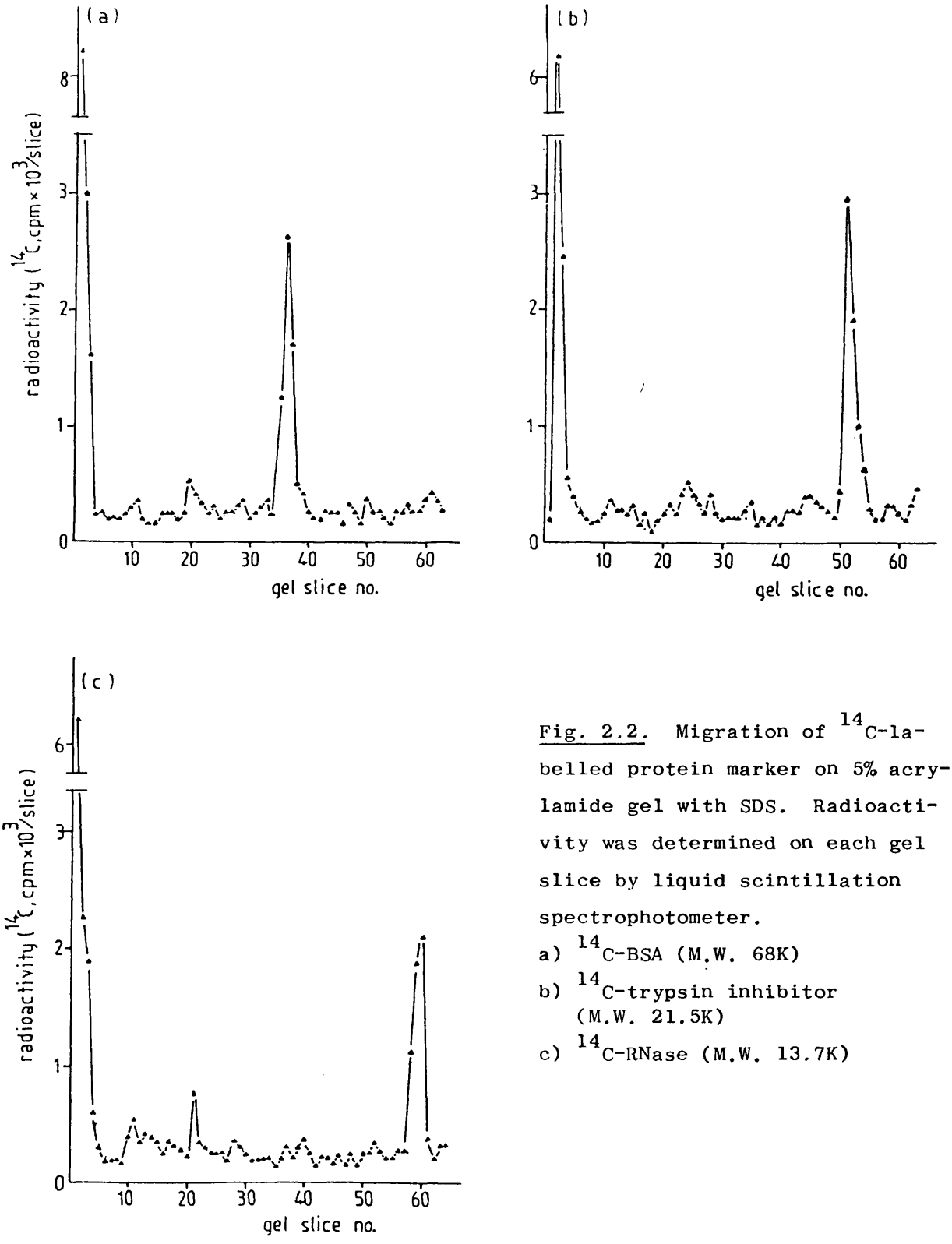


Fig. 2.2. Migration of ^{14}C-labelled protein marker on 5% acrylamide gel with SDS. Radioactivity was determined on each gel slice by liquid scintillation spectrophotometer.

a) ^{14}C-BSA (M.W. 68K)

b) ^{14}C-trypsin inhibitor (M.W. 21.5K)

c) ^{14}C-RNase (M.W. 13.7K)

phenol blue were measured. The gels were placed in staining solution (0.625g of Coomassie blue R250 in 250ml of methanol/glacial acetic acid/water at a ratio of 113/24/113) and left overnight at room temperature. The gels were destained in a mixture of methanol/glacial acetic acid/water (50/75/875) at 60°C to 70°C for 5-7 hr.

The Rf value of the cylindrical gel was determined for both samples and markers after scanning the gel at wavelength 560nm. The M.W. of protein was estimated from a calibration curve (M.W. versus Rf value).

The M.W. of protein in 5-15% gradient gel was determined by using a calibration curve that was plotted according to Poduslo and Rodbard (1980). The linear relationship between mobility and M.W. was shown in this calibration curve, which plotted log M.W. versus log% T (Fig 2.1.). The % T value was calculated from the migration distance of protein on the gel and the acrylamide concentration gradient. The degree of reproducibility of this method of protein markers is shown in Table 2.1.

Measuring the radioactivity of gel slices.

The method employed was adapted from the procedure of Aloyo,V.J. (1979). The unfixed gels were frozen at -70°C for 30 min and sliced into horizontal slices with a razor blade slicer. Each gel slice (about 1.5mm in length) was placed in 10ml of scintillation mixture consisting of hyamine hydroxide (1%,V/V), soluene 350 (1%, V/V) and PPO (0.6% W/V) in toluene, mixed well and left at room temperature for 24 hr. The level of radioactivity in the gel slices was determined with a Packard scintillation spectrophotometer. The graph of ^{14}C-protein marker containing gel slices is shown in Fig. 2.2.

By fluorography.

The fluorography method was shown to detect efficiently isotopes such as ^{3}H, ^{14}C, or ^{35}S, while autoradiography has been used to detect isotopes such as ^{125}I or ^{32}P. Two fluorographic methods for polyacrylamide gels were used in this study. The methods followed were those developed by Bonner and Laskey (1974) using PPO and DMSO and Skinner and Griswold (1983) using PPO and glacial acetic acid. Both methods detected radioactivity equally efficiently. But the acetic acid/PPO procedure had many advantages, including there is no need to pre-fix proteins in gels, used with either agarose or acrylamide gel, it is performed in a shorter time and with fewer toxic reagents than DMSO (Skinner and Griswold,1983).

In the PPO/DMSO procedure, the gel was fixed with a mixture of methanol/glacial acetic acid/water (113/24/113) for at least 1 hr. The gel was then dehydrated by soaking in 20 volumes of DMSO for 30 min followed by a second 30 min soaking in fresh DMSO. The gel was soaked for 3 hr in 20% (V/V) PPO in DMSO and the excess of DMSO was removed by washing with water for 1 hr.

In the acetic acid/PPO procedure, the gel was soaked in 20 volumes of glacial acetic acid for 5 min and then soaked in 20% (W /V) of PPO in glacial acetic acid for 1.5 hr. The gel was finally washed with water for 30 min before drying.

The gel was dried under vacuum by using a heated slab dryer, and exposed to X-ray film (Kodak X-Omat) at -70°C.

2.2.7.b. Two-dimensional separation of synthesized proteins.

The method of O'Farrell (1975) was used in two-dimensional (2D) gel analysis. Synthesized proteins were separated by isoelectric focusing in the first dimension and by SDS-gradient gel

electrophoresis in the second dimension. More than 1000 proteins can be resolved, and a protein as little as 10^{-4} to 10^{-5} of 1% of the total protein can be detected and quantified (O'Farrell 1975).

Isoelectrofocusing.

Synthesized proteins were focused in 4% acrylamide containing 9.2M urea, 2% carrier ampholyte (pH 3.5-10, Ampholine,LKB) 0.8ml of this 4% acrylamide was applied in a cylindrical glass tube (12cm long and 0.25cm diameter) and left for setting about 2 hr. This gel was prefocused at 200 volts for 15 min and 300 volts for 45 min to remove persulphate and isocyanate. Samples (20-100µl) containing 8M urea, 5% mercaptoethanol and 2% carrier ampholyte (pH 3.5-10) were applied and overlaid with 20µl of the overlay solution (3M urea and 1% carrier ampholyte (pH 3.5-10)). The electrode buffer (0.02M NaOH) was carefully applied over the overlay solution. The anolyte was 0.01M H_3PO_4 and the catholyte was 0.02M NaOH which were degassed before use. The IEF gels were focused at 500 volts for about 16 hr or until a total of at least 6000-9000 vh was achieved.

5 - 15% SDS-PAGE, the second dimension.

The IEF gel was equilibrated in a buffer consisting of 0.06M Tris-HCl pH 6.8, 3% SDS, 5% mercaptoethanol and 0.002% bromophenol blue for 1 hr and used immediately or kept at -70°C. The IEF gel was loaded on the 5-15% gradient acrylamide as a resolving gel with 3% acrylamide as a stacking gel and fixed in place with 1%agarose in 0.06M Tris-HCl pH6.8 and 1% SDS.

Electrophoresis and fluorography were performed in the same way as for single-dimensional gel.

Chapter Three Isolation, purification and characterization of poly(A)$^+$ RNA from tissues and cell lines.

3.1. Methods

3.1.1. Isolation of total RNA from tissues and cell lines.

3.1.1.a. Using guanidine-HCl, DTT and iodoacetate.

3.1.1.b. From isolated free and membrane bound polysome.

3.1.1.c. From isolated polysomal RNA and purified by sucrose gradient ultracentrifugation.

3.1.1.d. Using guanidinium thiocyanate.

3.1.1.e. Using phenol-chloroform deproteinization procedure.

3.1.2. Purification of poly(A)$^+$ RNA by oligo(dT) cellulose chromatography.

-Preparation of oligo(dT) cellulose column.

-Preparation of RNA.

-Separation of poly(A)$^+$RNA.

-Depletion of salt from RNA solution.

3.1.3. RNA determination.

-By extrinction coefficient.

-By orcinol reagent.

3.1.4. Radiolabelling of RNA in cell culture.

3.1.5. Gel electrophoresis of RNA.

3.1.5.a. Non-denaturing polyacrylamide gel.

-Preparation of gel.

-Preparation of RNA sample.

-Running and determination of the gel.

3.1.5.b. Agarose gel electrophoresis.

-Minigel electrophoresis.

-Fluorography of agarose gel.

-Agarose gel containing urea.

3.2. Results

3.2.1. Isolation and purification of poly(A)$^+$ RNA from tissues and cell lines and analysis of RNA translational activity in in vitro cell-free system.

3.2.1.a. Using guanidine-HCl, DTT and iodoacetate.

3.2.1.b. From isolated free and membrane bound polysome.

3.2.1.c. Purification of active RNA from polysomal RNA by sucrose gradient ultracentrifugation.

3.2.1.d. Using guanidinium thiocyanate.

3.2.1.e. Using phenol-chloroform deproteinization procedure.

3.2.2. Characterization of isolated RNA and synthesized protein by gel electrophoresis.

3.2.2.a. Characterization of isolated RNA from tissue and cells by gel electrophoresis.

i) Non-denaturing conditions in 4% acrylamide gel.

ii) Non-denaturing conditions in 3-8% SDS-PAGE.

iii) Radiolabelling of RNA in cells.

iv) Non-denaturing condition in 1.2% agarose minigels.

v) Denaturing conditions in 1.5% agarose gel in urea.

3.2.2.b. Characterization of synthesized protein by gel electrophoresis.

3.3. Conclusion.

3 Isolation, Purification and Characterization of poly(A)$^+$RNA from Tissues and Cell lines.

3.1. Methods

3.1.1. Isolation of total RNA from tissues and cell lines.

3.1.1.a. Using guanidine-HCl, DTT and iodoacetate.

The method employed was adapted from the procedure of Kahn et al.(1981). Tissues or washed cells were homogenized in 20 vol of prechilled (-20°C) homogenization buffer (20 mM sodium acetate buffer pH5 containing 7M guanidine-HCl,1mM DTT and 10mM iodoacetate) for 1 min with 10 strokes of a tight fitting Dounce glass homogenizer. The supernatant was separated by centrifugation at 10,000 rpm (11,000xg) for 30 min at -10°C and filtered through a sterile gauze. The supernatant was mixed with a half volume of prechilled (-20°C) 95% ethanol and the mixture was left at -20°C overnight. The precipitate was collected by centrifugation at 9,000 rpm for 30 min at -10°C, quickly dissolved at 4°C in a small volume of homogenization buffer containing 20mM sodium EDTA, mixed with a half volume of 95% ethanol and left at -20°C for 20 min. The precipitate was collected by centrifugation at 4,000 rpm for 10 min at -10°C. The precipitate was redissolved and precipitated again and this step was repeated twice. The final precipitate was dissolved in a minimum volume of 20mM sodium EDTA pH7 at 4°C and extracted with an equal volume of chloroform/isoamylalcohol (at a ratio of 24/1). The organic phase was re-extracted with a half volume of 20mM sodium EDTA pH7. The aqueous phases were pooled, mixed with a half volume of 95% ethanol and left overnight at -20°C. The RNA pellet was collected by centrifugation at 3,000 rpm for 10 min and washed twice with 3M Na(OAc) pH5, once with 66% ethanol and once with absolute ethanol, respectively. The RNA pellet was dried under N_2 gas and kept at -20°C.

3.1.1.b. From isolated free and membrane bound polysome.

This method was performed according to the method of Mechler and Rabbitts (1981). Tissues or washed cells were suspended in 5 vol of ice-cold hypotonic buffer medium RSB (10mM KCl, 1.5mM $MgCl_2$ 10mM Tris-HCl pH7.4) and then ruptured mechanically with 10 strokes of a tight-fitting Dounce glass homogenizer. The homogenate was diluted fivefold in 2.5M sucrose $TK_{150}M$ (0.15M KCl, 0,005M $MgCl_2$, 0.05M Tris-HCl pH7.4) and layered over 2 vol of 2.5M sucrose $TK_{150}M$. Another two layers of sucrose $TK_{150}M$ solution, 2.05M sucrose and 1.2M sucrose, were applied. This discontinuous sucrose gradient was centrifuged for 5 hr at 4°C in a Beckman SW 27 rotor at 25,000 rpm ($118,000xg_{max}$). The solution was fractionated into 30 drop fractions and the absorbance was measured at 260nm. Free polysomes were located in the loading zone while microsomes were at the interface between the 2.05M and 1.2M sucrose layer. These fractions were pooled and dialysed against 10% sucrose in $TK_{80}M$ (0.08M KCl, 0.005M $MgCl_2$, 0.05M Tris-HCl pH7.4), overnight. This solution was concentrated, treated with 0.5% sodium deoxycholate and 0.5% Brij 58 and layered over 15-30% sucrose gradient in $TK_{80}M$ with 69% sucrose in $TK_{80}M$ as a cushion solution. Centrifugation was carried out for 8.5 hr at 4°C in a Beckman SW 27 rotor at 27,000 rpm ($132,000xg_{max}$). The gradient was collected in fractions of 30 drops and the absorbance was measured at 260nm.

The polysome fractions were pooled, diluted with 1vol of water and mixed with equal volumesof hot SDS buffer (1% SDS, 0.2M NaCl,0.02M Tris-HCl pH7.4, 0.04M EDTA). The mixture was heated at 100°C for 120 sec, immediately plunged in ice for cooling to 30°C and adjusted to 0.1M Tris-HCl pH9 and 1% SDS. The mixture was extracted 3 times at room temperature with an equal volume of a

mixture of phenol, chloroform, and isoamylalcohol (50/50/1). The RNA pellet was precipitated from the aqueous phase by adding 0.1 vol of 2M Na(OAc) pH5.2 and 2.5 vol of ethanol and standing at -20°C overnight. The RNA pellet was collected by centrifugation at 12,500 rpm for 20 min at -10°C, washed twice with ethanol, dried and kept at -20°C.

3.1.1.c. From isolated polysomal RNA and purified by sucrose gradient ultracentrifugation.

This method was essentially performed as Taylor and Schimke (1973). Rat liver was resuspended in 10 vol of 0.25M sucrose in buffer B (50mM Tris-HCl pH7.7, 25mM NaCl, 5mM $MgCl_2$ and 500µg/ml Na-heparin), homogenized with 5 strokes at 1,000 rpm in Dounce homogenizer and centrifuged at 9,500 rpm for 10 min at 4°C. 9 vol of supernatant was diluted with 1 vol of 10% triton X-100 and 10% sodium deoxycholate, mixed in a homogenizer with a tight pestle for 4 strokes and layered over a step gradient of sucrose in buffer B (0.5M sucrose, 1M sucrose and 2.5M sucrose). The gradient was centrifuged for 2.5 hr in Beckman SW 27 rotor at 27,000 rpm ($132.000 \times g_{max}$) and collected in fractions of 30 drops. The polysome fraction was collected and diluted with 4 vol of buffer B. The supernatant was separated by centrifugation at 12,000 rpm ($14,600 \times g_{max}$) for 5 min at 4°C and made to 1% SDS, 50mM sodium EDTA pH7 and 100mM NaCl. The RNA pellet was precipitated by adding 2 vol of ethanol and standing at -20°C for at least 8 hr. It was collected by centrifugation at 12,000 rpm for 15 min at 0°C, dissolved in buffer C (25mM Tris-HCl pH7.4, 2mM sodium EDTA, 1% SDS) and layered over 5-20% linear sucrose gradient in buffer C. Centrifugation was carried out at 27,000 rpm in Beckman SW 27 rotor for 18 hr. The gradient was fractionated into 30 drops per tube and the absorbance was measured at 260 nm. The 28S and 18S RNA fractions were pooled

and mixed with a half volume of water and 2 vol of ethanol. After 18 hr at -20°C, the precipitate was collected by centrifugation and washed once with 3M Na(OAc) pH6 containing 5mM EDTA, once with 70% ethanol with 2M NaCl and once with absolute ethanol, dried under N_2 gas and kept at -20°C.

3.1.1.d. By using guanidinium thiocyanate.

This method was performed according to the method of Chirgwin et al. (1979). 10g of rat liver was homogenized in 100ml of guanidinium thiocynate stock solution(see note 2)in an MSE homogenizer at full speed. The supernatant was separated by centrifugation at 8,000 rpm for 10 min at 10°C, mixed with 0.025 vol of 1M acetic acid and 0.75 vol of absolute ethanol and left at -20°C overnight. The precipitate was collected by centrifugation at 6,000 rpm for 10 min at -10°C and resuspended in 0.5 vol of guanidine hydrochloride stock solution (see note 2). The mixture was shaken vigorously and warmed to 68°C to ensure complete dispersion of pellet, then it was reprecipitated in 0.025 vol of 1M acetic acid and 0.5 vol of ethanol. After 3 hr at -20°C, the precipitate was collected by centrifugation at 6,000 rpm for 10 min at -10°C. The precipitate was resuspended and reprecipitated again. The final precipitate was mixed with ethanol to extract excess guanidine hydrochloride and collected by centrifugation at 6,000 rpm for 5 min at -10°C. The precipitate was dried in a stream of N_2 and dissolved in sterile water at 1ml/g of tissue. The supernatant was collected by centrifugation at 13,000 rpm for 10 min at 10°C and insoluble material was re-extracted with sterile water (0.5ml/g tissue). The supernatants were pooled, mixed with 1 vol of 2M K(OAc) pH5 and 2 vol of absolute

ethanol and left at -20°C, overnight. The pellet was collected, washed with 95% ethanol, dried in a stream of N_2 and kept at -20°C.

Note 1). Guanidinium thiocyanate stock solution was prepared by dissolving 5g of guanidinium thiocyanate and 0.5g of sodium-N-lauroylsarcosine with 0.7ml of 2-mercaptoethanol, 0.33ml of 30% antifoam A and 2.5ml of 1M Na(OAc) pH7.0 with warming and stirring. The solution was adjusted to 100ml, filtered and titrated to pH7.0 with 1N NaOH.

Note 2). Guanidine hydrochloride stock solution was prepared by mixing 50 ml of 7.5M guanidine HCl pH7.0 with 1.25ml of 1M sodium citrate pH7 and 0.03g of DTT.

3.1.1.e. Using a phenol-chloroform deproteinization procedure

The method of Noyes et al. (1979) was used with some modification. 3g of tissue or washed cells were suspended in 16ml of a mixture solution of phenol saturated with buffer A (0.2M Tris-HCl pH9, 0.1M LiCl, 25mM EDTA and 1% SDS), chloroform and isoamylalcohol (at a ratio of 50/48/2) and mixed with 16ml of buffer A. The mixture was homogenized at high speed in an MSE homogenizer for 2 min. The aqueous phase was separated by centrifugation at 11,000 rpm (15,000xg) for 15 min and the interphase and organic phase were re-extracted with 16ml of buffer A. The aqueous phases were pooled and re-extracted with 8ml of phenol (saturated with buffer A)/chloroform/isoamylalcohol (50/48/2). The nucleic acid was precipitated from the aqueous phase by adding 0.1 vol of 2M Na(OAc) and 2.5 vol of 95% ethanol. After standing at -20°C overnight, the pellet was collected, washed twice with 70% ethanol, dried and dissolved in sterile water.

The separation of DNA fron total nucleic acid isolated from tissue was performed by adding 1% streptomycin sulphate pH5 to nucleic acid until no more DNA precipitated. The supernatant was separated after 6 hr at 4°C by centrifugation and mixed with 0.1 vol of 2M Na(OAc) and 2.5 vol of 95% ethanol. After leaving at -20°C overnight, the RNA pellet was collected by centrifugation at 10,000 rpm for 20 min at -10°C, washed with 95% ethanol, dried in a stream of N_2 and kept at -70°C.

The isolated nucleic acid from cell lines was mixed with 0.25 vol of 10M LiCl and left at 0°C for 1 hr. The RNA pellet was collected by centrifugation at 10,000 rpm for 20 min at 0°C, washed twice with 2 vol of cold 75% ethanol, dried and kept at -70°C. A similar method for RNA precipitation from total nucleic acid solution has been used by Duguid et al. (1976).

3.1.2. Purification of poly(A)$^+$RNA by oligo(dT) cellulose chromatography.

The method employed was adapted from the procedure of Aviv and Leder (1972). There are two procedures used in purification of poly(A)$^+$RNA from tissues and cell lines, one cycle and two cycles of chromatography. A similar method for purification of poly(A)$^+$RNA by two cycles of oligo(dT) cellulose chromatography has been employed by Noyes et al. (1979).

Preparation of oligo(dT) cellulose column.

0.25g of oligo(dT) cellulose was suspended in 10mM Tris-HCl pH7.4 and packed into a column (1 x 5cm). The new column was treated with E. coli tRNA to reduce non-specific binding. The column was firstly equilibrated with binding buffer (0.5M LiCl, 0.2% SDS and 10mM Tris-HCl pH7.4) and $3A_{260}$ units of E.coli tRNA

was then applied. The column was continuously washed with 30ml of binding buffer or until the absorbance of the effluent at 260nm was less than 0.01.

The efficiency of binding of the new column was checked by using poly(A). The binding of poly (A) by oligo(dT) cellulose from Sigma and BRL was found to be 80-85% and 45-50%, respectively. The amount of poly(A) was 60-80 A_{260} units/g oligo(dT) cellulose.

Preparation of RNA.

RNA pellets were dissolved in binding buffer,incubated at 60-70°C for 2-5 min and immediately cooled in ice. The clear supernatant was collected by centrifugation at 4,000 rpm for 10 min.

Separation of poly(A)$^+$RNA.

The RNA sample in binding buffer at concentration of 50-100 A_{260}units was applied to a 1ml (about 0.25g, dry weight) column of oligo(dT) cellulose, previously equilibrated with the same buffer. RNA solution was passed through the column 3 times. The unbound material was eluted by continued washing with binding buffer until the absorbance at 260nm was less than 0.01. The column was then washed with buffer containing 0.1M LiCl, 0.2% SDS and 10mM Tris-HCl pH7.4,although this step was not performed when poly(A)$^+$RNA was purified by two cycles of olig(dT) cellulose chromatography. The bound material (poly(A)$^+$RNA) was then eluted with 10mM Tris-HCl pH 7.4 or 10mM N-ethylmorpholine pH7.4 (elution buffer).

The bound fractions were pooled, lyophilized, dissolved in a small volume of sterile water and poly(A)$^+$RNA was precipitated by the addition of 0.1 vol of 20% Na(OAc) plus 2 vol of ethanol. After keeping at -20°C overnight, the poly(A)$^+$RNA precipitate was

collected by centrifugation at 12,500 rpm for 1 hr at -10°C, washed with ethanol, dried in a stream of N_2 and dissolved in sterile water (at a concentration of 0.02-0.1 A_{260}units/µl).

For the second cycle of oligo(dT) cellulose chromatography, the bound material from the first oligo(dT) cellulose column was adjusted with LiCl and SDS to the same concentration as binding buffer. This bound material was applied to a 1ml of oligo(dT) cellulose column (a new column) and passed through the column three times. The unbound material was eluted by continuous washing with the binding buffer. The bound material was then eluted from the column with elution buffer. The poly(A)$^+$RNA was precipitated as described above.

The absorption capacity of oligo(dT) cellulose for RNA is dependent on the salt concentration. The poly(A)$^+$RNA was eluted from the column at low salt concentration. The quantity of poly(A)$^+$RNA from tissues or cell lines adsorbed and eluted from the column was between 0.3 and 1.0% of total RNA for one cycle of oligo(dT) cellulose chromatography. The second bound fraction of poly(A)$^+$RNA was about 15-30% of the amount of poly(A)$^+$RNA from first bound fraction. The elution profiles for poly(A)$^+$RNA that was separated by one cycle and two cycles of oligo(dT) cellulose chromatography are shown in Fig 3.1(a) and 3.1.(b) and (c), respectively.

Depletion of salt from RNA solution.

High salt concentration had been shown to have an effect on translation in cell free system (Weber et al.,1977; Ranu and Bhala, 1981) and a small volume of poly(A)$^+$RNA was achieved from cell lines or tissue isolation. Therefore a mini system to deplete salt which gave high recovery of RNA was adapted from the method used by Tuszynski et al. (1980).

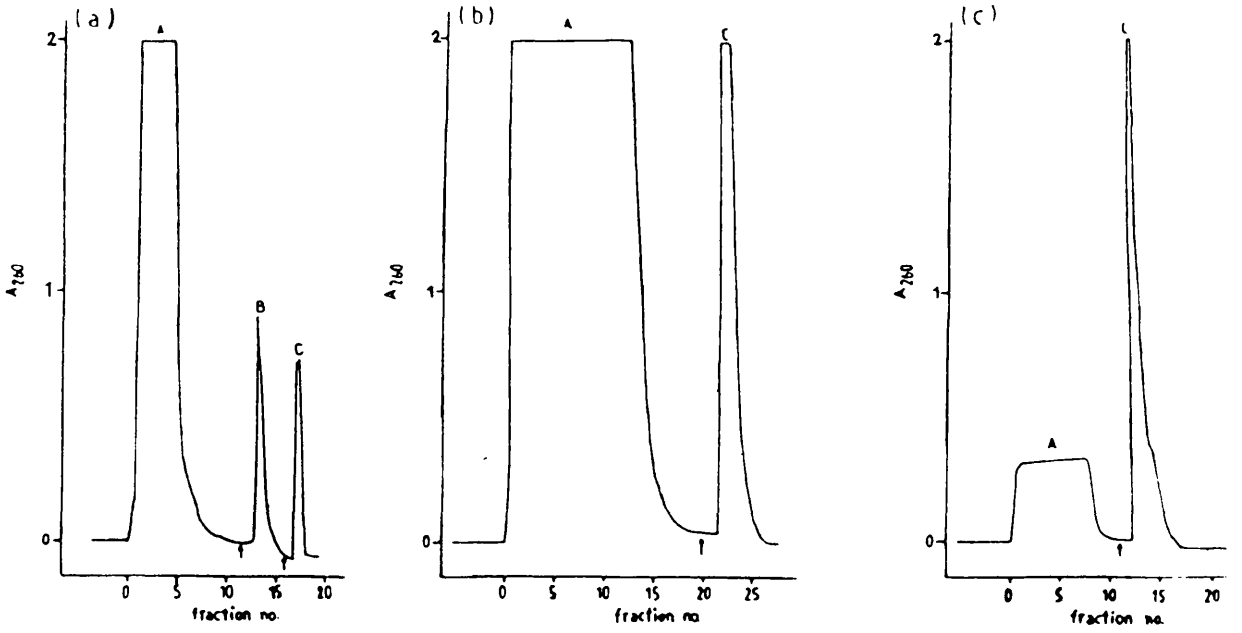


Fig. 3.1. Oligo(dT) cellulose chromatography of poly(A)$^+$RNA. RNA was dissolved in binding buffer (0.5M LiCl, 10mM Tris-HCl pH7.4, 0.2% SDS) and applied to the column. The unbound fraction (A) was a material that was not retained by the column. Material (B) was eluted with buffer (0.1M LiCl, 10mM Tris-HCl pH7.4, 0.2% SDS). The poly(A)$^+$RNA$_{(C)}$ was eluted by elution buffer (10mM Tris-HCl pH7.4 or 10mM N-ethylmorphorine pH7.4).

a) Total RNA (67.5 A_{260}units) was extracted from NS1 cells by phenol-chloroform deproteinization procedure and poly(A)$^+$RNA was separated by one cycle of oligo(dT) cellulose chromatography. A(55.8A_{260}units; A_{260}/A_{280}= 2.08); B(1.6A_{260}units; A_{260}/A_{280}=2.12), C(2.23A_{260}units, A_{260}/A_{280}=2.3)

b&c) Two cycle of oligo(dT) cellulose chromatography were used to purified poly(A)$^+$RNA from total rat liver RNA (355 A_{260}units) extracted by phenol-chloroform deproteinization procedure.
In (b): A(304A_{260}units, A_{260}/A_{280}=2.02) and C(9.83A_{260}units, A_{260}/A_{280}=2.3)
in (c): A(4.57A_{260}units, A_{260}/A_{280}=1.79) and C(4.17A_{260}units, A_{260}/A_{280}=2.29)

An Eppendorf tip for 10-250µl was cut at the end to make a larger hole, plugged with glasswool and then filled with Sephadex G-25 (medium) which was suspended in sterile water. The column was packed and the water dried out by centrifugation at 1,000 rpm for 2 min. The column was then inserted into a collection tube, 5x35mm (Luckham LP2 tubes). The RNA sample (about 100-500µl) was applied to the column and centrifuged at 1,000 rpm for 2 min. High percentage recovery of RNA was achieved while at least 90% of salt was removed by this method. The RNA solution was aliquoted and kept at -70°C.

3.1.3. RNA determination.

by extinction coefficient

The concentration of RNA was determined spectrophotometrically by the absorption at wavelength 260nm. An absorbance of 1 corresponded to approximately 40µg RNA/ml(Maniatis et al.,1982;Mechler and Rabbitts,1981) or 50µg/ml (Kahn et al.,1981) of RNA.

by orcinol reagent.

100µl of RNA solution was made up to 1ml with distilled water and mixed with 2 ml of orcinol reagent (0.1% orcinol, 0.5% $FeCl_3$ in concentrated HCl). The mixture was heated in a water bath at 90°C for 30 min amd cooled under tap water before measuring the absorbance at 665nm. The concentration of RNA was determined on a calibration curve using standard yeast RNA. The range of RNA concentration was between 30 and 75µg/ml.

3.1.4. Radiolabelling of RNA in cell culture.

K562 and HMy2 cells with viability more than 90% were washed and suspended in fresh culture medium at a density of

0.7-1x10^6 cells/ml. ^{3}H-uridine(3µCi) was added and the incubation was continued at 37^{o}C in an atmosphere of 5% CO_2. Triplicate samples (100µl) were taken at hourly intervals, filtered using GF/C filter (Whatman) and washed with 200µl of PBS, 5ml of 10%TCA, 5ml of 5%TCA and 10ml of 95% ethanol,respectively. The GF/C filter was dried and radioactivity was counted in 3ml of scintillation fluid. The rest of the cells were harvested at 4 hr incubation, washed twice with PBS and used to isolate RNA. The uptake of ^{3}H-uridineby these cell lines was dependent on incubation time as shown in Fig 3.2.

3.1.5. Gel electrophoresis of RNA.

3.1.5.a. Non-denaturing of polyacrylamide gel.

Preparation of gel.

Cylindrical gels in glass tubes (10x0.5cm) contained 4% acrylamide, which was prepared by mixing 1.3ml of 30.8% acrylamide and N,N-methylene bisacrylamide with 3.3ml of gel buffer (5,99mM EDTA, 0.06M Na(OAc), 0.12M Tris-HCl pH7.8), 10µl of TEMED, 0.1ml of 1% ammonium persulphate and 5.3ml of water. The gel solution was de-gassed and poured into glass tube. After setting for 1 hr, the gel was prerun at constant current (about 5mA/tube) for 1 hr.

In vertical slab gels (14X16cm with 1mm thickness), 3-8% gradient polyacrylamide gel were used as a resolving gel with 3% poly-acrylamide as a stacking gel.

Preparation of RNA sample.

50-200µg of RNA pellet was dissolved in 100µl of electro-phoresis buffer (2mM EDTA, 0.02M Na(OAc), 0.04mM Tris-HCl pH7.8 and 0.5% SDS). The clear supernatant was collected from centrifugation and mixed with 10µl of 5% bromophenol blue in 50% glycerol or in 30% Ficoll.

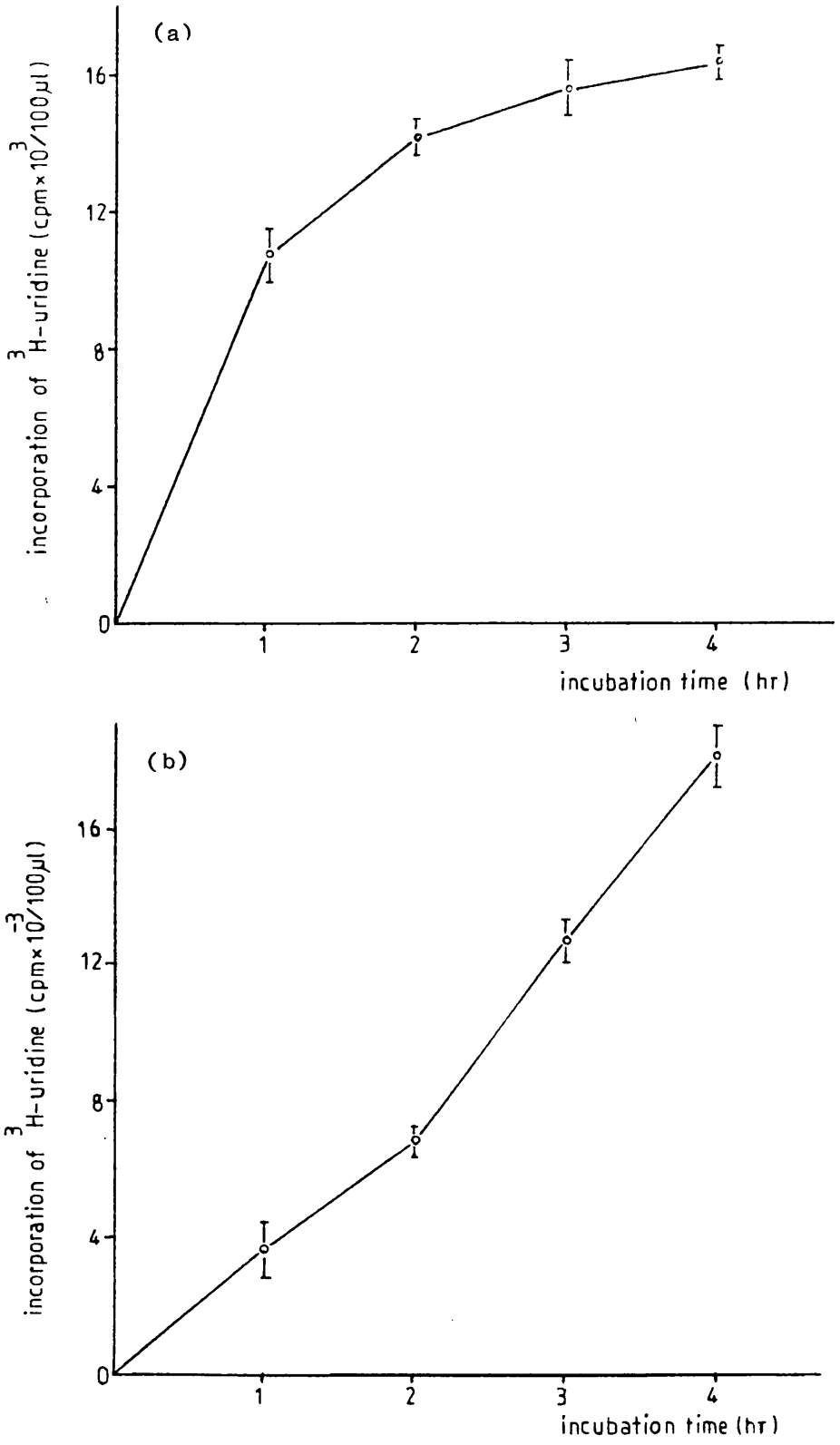


Fig. 3.2. Time course of ^{3}H-uridine incorporation by K562 cell (a) and HMy2 cell (b). The results are expressed as cpm/100µl of sample that contained 75,000 cells for K562 cell and 100,000 cells for HMy2 cell.

Running and determination of the gel.

The two compartments of the electrophoresis apparatus were filled with electrophoresis buffer. Electrophoresis was performed at a constant current (about 5mA/tube) until bromophenol blue moved nearly to the end of the cylindrical gel. For slab gels, the electrophoresis was performed at constant voltage of 200volts for 15 min and decreased to 150 voltsfor approximately 2 hr.

The cylindrical gels were washed with water and scanned at 260nm using a Pye Unicam UV-spectrophotometer (slit width 1.5mm).

Both ethidium bromide and acridine orange were used to stain nucleic acid in the gel. Acridine orange can differentiate single- and double-stranded nucleic acid (Carmichael and Mc Master, 1980). The gel was washed with 0.1N $NH_4(OAc)$ for 1 hr and stained with ethidium bromide (1µg/ml in 0.1N $NH_4(OAc)$) for at least 1 hr before locating the RNA band under short wavelength UV light (Sharp et al., 1973). After 30 min staining of the gel with acridine orange (30µg/ml of 10mM phosphate buffer pH7), the gel was washed with 10mM phosphate buffer pH7 for 1 hr before detecting the RNA band under a short wavelength UV light.

3.1.5.b. Agarose gel electrophoresis.

Minigel electrophoresis.

The horizontal, submerged gel apparatus of minigel type was manufactured by Cambridge Biotechnology Lab. 12ml of 1.2% agarose gel in electrophoresis buffer (0.09M Tris, 2.55mM EDTA and 0.09M boric acid, pH7.9) was used for one gel and left for setting about 30 min. 2-10µl RNA samples were applied and 25ml of electrophoresis buffer was added to submerge the gel. Electrophoresis was performed at 100 volts for 10 min and reduced to 80 volts for about 30 min or until the bromophenol blue moved a distance of 5-5.5cm

(total length of gel was 6cm). The gel was stained with ethidium bromide or fluorographed.

Fluorography of agarose gel.

The agarose gel was fixed in 1% streptomycin sulphate, overnight at room temperaure. The gel was then dehydrated in 20 vol of methanol for 30 min, twice, and soaked in 10% PPO (W/W) in methanol for 3 hr. The gel was dried at room temperature and exposed to X-ray film (Kodak X-Omat) at -70°C. This is similar to the method used by Lasky and Mills (1975).

Agarose gel containing urea.

The method was performed according to Rosen et al.(1975) with some modification. 1.5% agarose gel was prepared by dissolving 36g of urea and 1.5 g of agarose in 10ml of 0.25M trisodium citrate and 63ml of water. 40ml of this agarose solution was used to prepare a slab gel, (12 X 16cm with 1.5mm thickness). 5-20µg of RNA was dissolved in 8µl of sample buffer (7.8M urea in 25mM citrate buffer pH3.5) with 2µl of 5% bromophenol blue in 30% Ficoll. Horizontal gel electrophoresis was performed at a constant voltage of 200 volts for 30 min and increased to 300 volts for 4-5 hr. The electrophoresis buffer was 25mM citrate buffer pH3.5.RNA was detected by staining or fluorography.

3.2. Results.

The ability to isolate, examine, and quantitate individual poly(A)$^+$RNA or mRNA from eukaryotic cells is a tool for analysis of transcriptional and translational control mechanisms. Procedures available for the detection, isolation, and characterization of eukaryotic mRNA have involved the use of cell-free translation system

in conjunction with the electrophoretic assays to identify protein products. Many procedures have been used to isolate total RNA from a variety of tissues and cell lines. The aim of the study was the attempt to prepare an active and undegraded RNA which can be translated to full-length polypeptide chains in a cell-free system.

The presence of poly A in most mRNA provides a precise criterion for the identification and purification of poly(A)$^+$RNA. Several procedures have been developed using the complementary base pairing property of poly A. Oligo(dT) coupled to cellulose is an effective adsorbent for poly(A)$^+$RNA. It has been used successfully for the isolation of many eukaryotic mRNAs on a relatively large scale. Therefore this affinity chromatography was chosen to purify poly(A)$^+$RNA from isolated total RNA. These total RNA and poly(A)$^+$RNA were further analysed on gel electrophoresis, either polyacrylamide or agarose gel.

Translation of an isolated RNA and poly(A)$^+$RNA in cell-free protein synthesizing system is a useful criterion for detection and characterization of purified RNA. Wheat germ lysates were firstly used in these experiments because of their low endogenous protein synthesis and high efficiency in translation of many eukaryotic and viral mRNAs. Wheat germ lysate was shown to be active in translation of poly u and TMV RNA which is described in detail in chapter 4, section 4.1.1. Using the same optimal conditions, this wheat germ lysate cell-free system was used to check the activity of isolated total RNA or poly(A)$^+$RNA from tissues and cell lines. In addition, the rabbit reticulocyte lysate (nuclease treated) cell-free system which was active and probably gave the highest proportion of full-length polypeptides was also used in these experiments. Some translated products were further characterized in SDS-PAGE.

Table 3.1. Purification and recovery of RNA and poly(A)$^+$RNA from tissues and cell lines using guanidine-HCl.

Source and amount	Yield of total RNA(mg)	Ratio of A_{260}/A_{280} of RNA	% Yield (RNA/g tissue)	Yield of poly(A)$^+$ RNA(A_{260} units)	% Yield (poly(A)$^+$RNA/total RNA)
Daudi cells (2.8×10^8)	0.049	1.60	0.004	-	-
Rat liver (4g)	0.681	1.78	0.017	0.03	0.67
Rat liver (10g)	2.325	1.90	0.023	0.60	0.645
Rat muscle (2g)	0.263	2.16	0.013	0.005	0.625

3.2.1. Isolation and purification of poly(A)$^+$RNA from tissues and cell lines and analysis of RNA translational acivity in in vitro cell-free system.

The results of isolation and characterization of total RNA or poly(A)$^+$RNA from tissues or cells will be discussed separately for each isolation method.

3.2.1.a. Using guanidine -HCl, DTT and iodoacetate.

Total cellular high M.W. RNAs were isolated by homogenization of cells or tissue in guanidine-HCl (a powerful denaturing agent) with DTT and iodoacetate, (inhibitors of RNase activity) as described in detail in Method;3.1.1.a.

Daudi cells, rat liver and rat muscle were used in the isolation of RNA and the yield of RNA varied from 0.004% to 0.023%. The ratio of A_{260}/A_{280} was 1.6 to 2.16. This ratio provides an estimate of the purity of the nucleic acid,and pure preparations of RNA have A_{260}/A_{280} of approximately 2.0(Maniatis et al.,1982) or 1.8-2.0 (Kahn et al.,1981). Therefore,these RNA pellets were mainly nucleic acid. The RNA was further purified for poly(A)$^+$RNA by using oligo(dT) cellulose column, the average final yield of poly(A)$^+$RNA was 0.647±0.018% of total RNA. The summary of these results is presented in Table 3.1.

The translational activity of both total RNA and poly(A)$^+$RNA was characterized in wheat germ lysate cell-free system. The RNA template activity was estimated by measuring ^{3}H-Phe incorporation. Total RNA from Daudi cells and rat muscle gave no incorporation of ^{3}H-Phe and the incorporation of ^{3}H-Phe was increased 1.65 fold when using poly(A)$^+$RNA from rat muscle at a concentration of 0.05 A_{260} units in 25µl reaction mixture. For rat liver, maximum increase in incorporation of radioactivity into protein was only 1.6 fold for total RNA at a concentration of 10µg in 25µl reaction mixture and about

2.6 to 3.3 fold for poly(A)$^+$RNA at a concentration of 0.03 - 0.2 A_{260} units in 25µl reaction mixture. The purified poly(A)$^+$RNA gave the highest incorporation of radioactivity but this value was still very low when compared to standard poly u which gave an increase in incorporation of ^{3}H-Phe of about 10 fold at a concentration of 20µg in 25µl reaction mixture. By this method the maximum incorporation of radioactivity into protein was not obtained for either total RNA or poly(A)$^+$RNA. Even poly(A)$^+$RNA, which gave higher radioactivity incorporation, did not give reproducible results, probably a result of unstable or degraded RNA. Therefore, the rapid large-scale isolation of polysomes from fresh tissues or cells was used to obtain large quantities of undegraded and biologically active mRNA(Mechler and Rabbitts,1981).

3.2.1.b. From isolated free-and membrane-bound polysome.

In this method, cells or tissues were broken by Dounce homogenization. The post nuclear supernatant at a final concentration of 2.1M sucrose were fractionated to yield free ribosomal and microsomal fractions on a discontinuous sucrose density gradient (2.5M and 2.05M sucrose) as shown in Fig 3.3. In rat liver,rat muscle, calf thymus and Daudi cells, the average distribution of RNA (determined by A_{260}) between the free ribosomal (fraction no. 6-12) and microsomal (fraction no. 16-19) fractions was 23.7 $\pm$ 6.17 % and 76.29 $\pm$ 6.17%, respectively.

Polysomes were further separated from the free-ribosomal and microsomal fractions by sedimentation on 15-30% sucrose density gradient with 69% sucrose cushion (Fig. 3.4). The recovery of the polysomal fraction was approximately 32%. The total RNA was extracted from the polysomal fraction and the percentage yield varied from 0.44 to 1.18. Moreover the average yield of poly(A)$^+$RNA after passing the

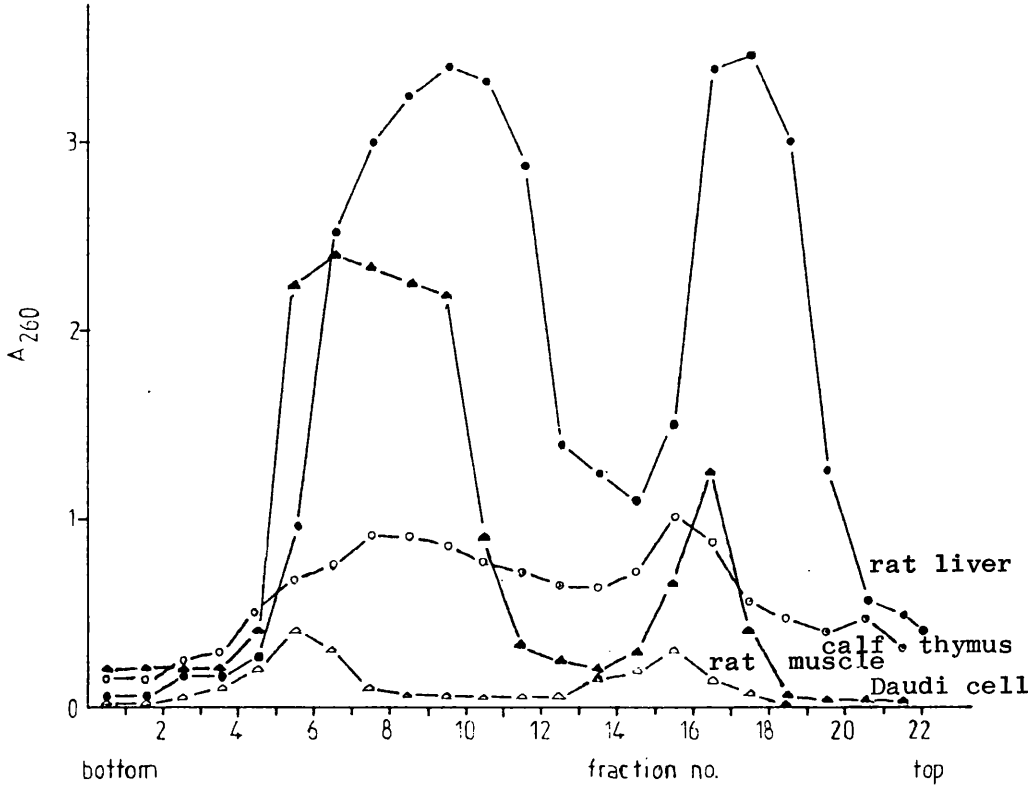


Fig. 3.3. Sedimentation pattern of free ribosomal and microsomal fractions of the cytoplasmic extract. Samples were layered on discontinuous sucrose density gradient and were centrifuged for 5 hr at 25,000 rpm. in Beckman SW27 rotor. Gradients were collected in 20-22 fractions and absorbance was measured at 260 nm.

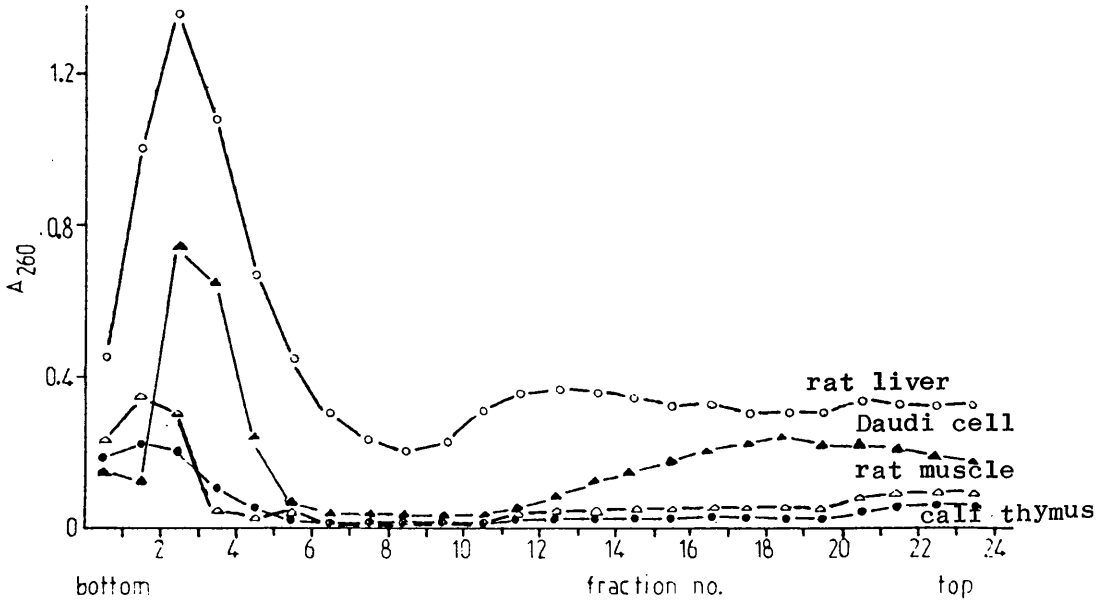


Fig. 3.4. Sedimentation profiles of polysome on 15-30% sucrose density gradients. Centrifugation was carried out for 8.5 hr at 27,000 rpm. in Beckman SW27 rotor at 4°C. Gradients were collected in 24 fractions and the absorbance was measured at 260 nm.

Table 3.2. The yield of total RNA and poly(A)$^+$RNA isolated from polysomes of tissues and cells.

Source and amount	Yield of total RNA(mg)	% Yield (RNA/g tissue)	Yield of poly(A)$^+$ RNA(A_{260} units)	% Yield (poly(A)$^+$RNA/total RNA)
Daudi cells (2.3×10^8)	5.02	0.44	0.23	1.17
Rat muscle (1g)	11.85	1.18	0.40	0.84
Rat liver (1g)	6.15	0.61	0.26	0.97
Rat liver (3g)	14.00	0.47	0.36	0.65
Calf thymus (2g)	1.70	0.09	-	-

total RNA through oligo(dT) cellulose column was 0.908 ± 0.19% of total RNA. The variation of the RNA yield probably related to the type of tissue or cell, containing different amounts of RNA. The yield of RNA at each step in the procedure is summarized in Table 3.2.

By examination of their translational properties in wheat germ lysate cell-free system, poly(A)$^+$RNA gave higher incorporation than total RNA. Very low incorporation of ^{3}H-Phe (about 1 fold) was obtained when using total RNA or poly(A)$^+$RNA from Daudi cells and calf thymus. For rat liver and rat muscle, the maximum incorporation of radioactivity into protein was obtained from RNA concentrations in the total translation reaction mixture of 16 to 40 A_{260} units/ml, for total RNA and about 0.2-0.4 A_{260} units/ml for poly(A)$^+$RNA. Maximum stimulation was 1-2 fold with total RNA and 2.4-3.5 fold with poly(A)$^+$ RNA.

The incorporation of ^{3}H-Phe was still lower than standard poly u and was not proportional to the amount of RNA added. This method gave a higher yield of total RNA after extraction from polysomes but these RNAs still had low activity in in vitro translation system. This procedure involved many steps in the purification of polysome and required a longer time to get the final poly(A)$^+$RNA product. It is possible that the RNAs may have lost their activity by the contamination with RNase, or other factors during the step of preparation, thus producing degraded RNA (Lomedico and Saunders, 1976).

The yield of poly(A)$^+$RNA was very low and total RNA had lower activity in translation in wheat germ systems than poly(A)$^+$RNA. Therefore, partial purification of 18S RNA from total rat liver polysomal RNA were used instead of these two types of RNA (Taylor and Schimke, 1973; Bantle et al., 1976).

3.2.1.c. Purification of active RNA from polysomal RNA by sucrose gradient ultracentrifugation.

This procedure was performed according to the method of Taylor and Schimke (1973). They found that rat liver polysomal RNA, which had a sedimentation coefficient of approximately 18S in sucrose gradient, could direct the synthesis of albumin in a rabbit reticulocyte lysate cell-free system.

After homogenization of the rat liver in the presence of triton X-100 and sodium deoxycholate, the polysomes were separated on a discontinuous sucrose gradient. The polysomal fraction was collected at the interface between the 1M and 2.5M sucrose layer and the RNA was precipitated out using ethanol. The RNA pellet was dissolved in buffer C, loaded on a linear 5-20% sucrose gradient and centrifuged at 25,000 rpm ($118,000 \times g_{max}$) for 18 hr. Two major peaks were observed at the sedimentation coefficients of approximately 28S and 18S as shown in Fig. 3.5. The yield of 18S RNA was approximately 5% of total polysomal RNA. Scanning the absorbance at wavelengths between 340nm and 180nm demonstrated that, the maximum absorbance was given at 260nm and 188nm and the average ratio of A_{260}/A_{280} was 2.09 ± 0.05. indicating low protein contamination. The absorbance profile of these RNAs is shown in Fig. 3.6.

The incorporation of ^{3}H-Phe in the wheat germ lysate was related to RNA concentration and the maximum radioactivity incorporation into protein was about 4 fold above control (without mRNA) at a concentration of 30µg of 18S RNA in 25µl reaction mixture (Fig. 3.7.a.). A similar result was given when using rabbit globin mRNA (commercial product from BRL) as a template. The maximum incorporation of ^{3}H-Phe was about 3.5 fold greater than a mRNA free control at a concentration of 0.1µg in 25µl reaction mixture (Fig.3.7.b). These results suggested that the wheat germ lysate cell -free system

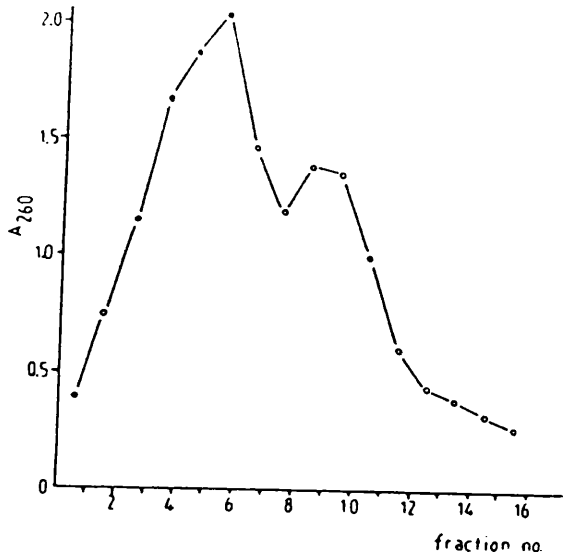


Fig. 3.5. Sucrose gradient sedimentation profile of polysomal RNA from rat liver. 2-5mg of polysomal RNA sedimented in a linear 5-20% sucrose gradient. Ultracentrifugation was performed in Beckman SW27 rotor at 25,000 rpm. for 18 hr. 1ml fraction was collected and absorbance was measured at 260 nm.

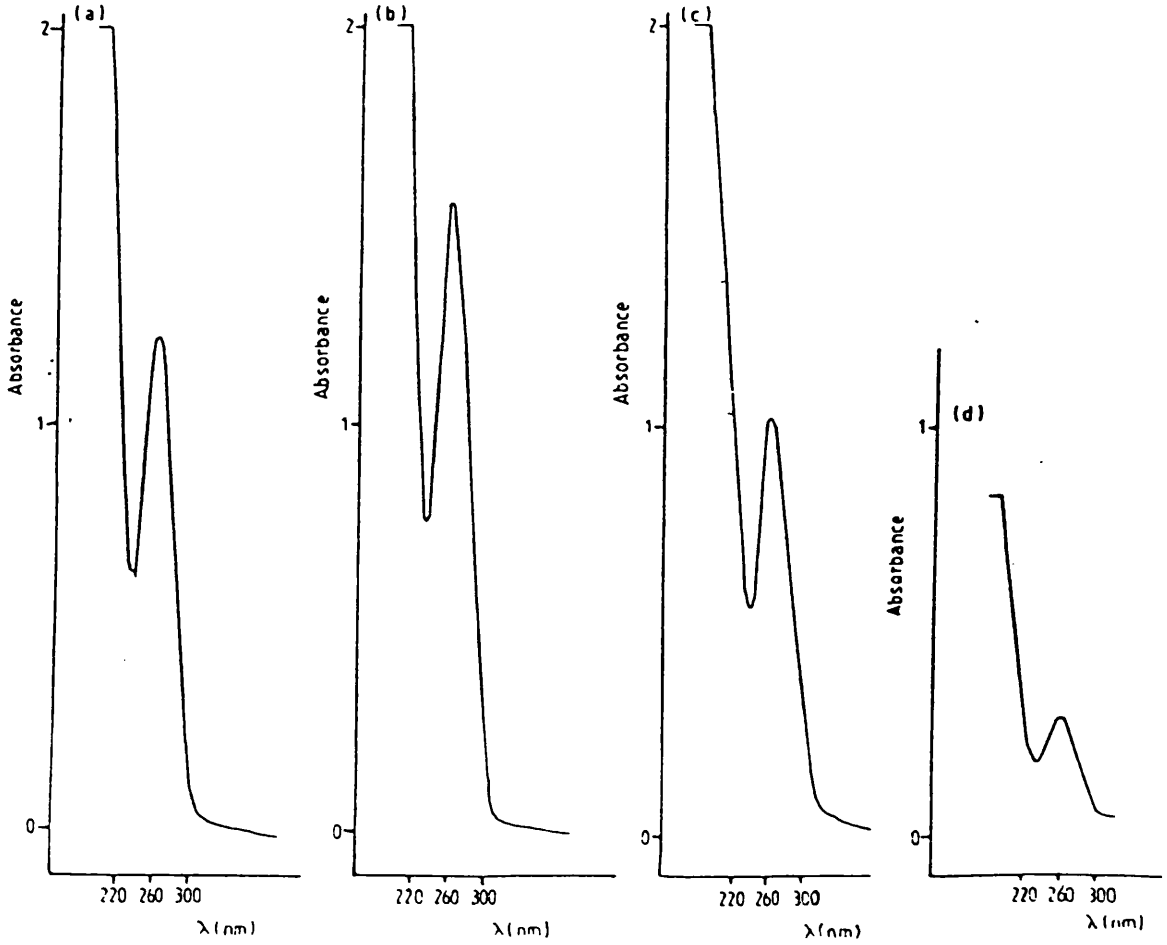


Fig. 3.6. Spectra profile of the RNA. The absorbance was measured at wavelengths from 340 to 180 nm. (a) rat liver RNA, (b) calf thymus RNA, (c) rat liver 18S RNA, (d) rat liver 28S RNA. These RNAs were prepared (as method c,3.1.1.c.) and separated for 18S and 28S by sucrose density gradient centrifugation.

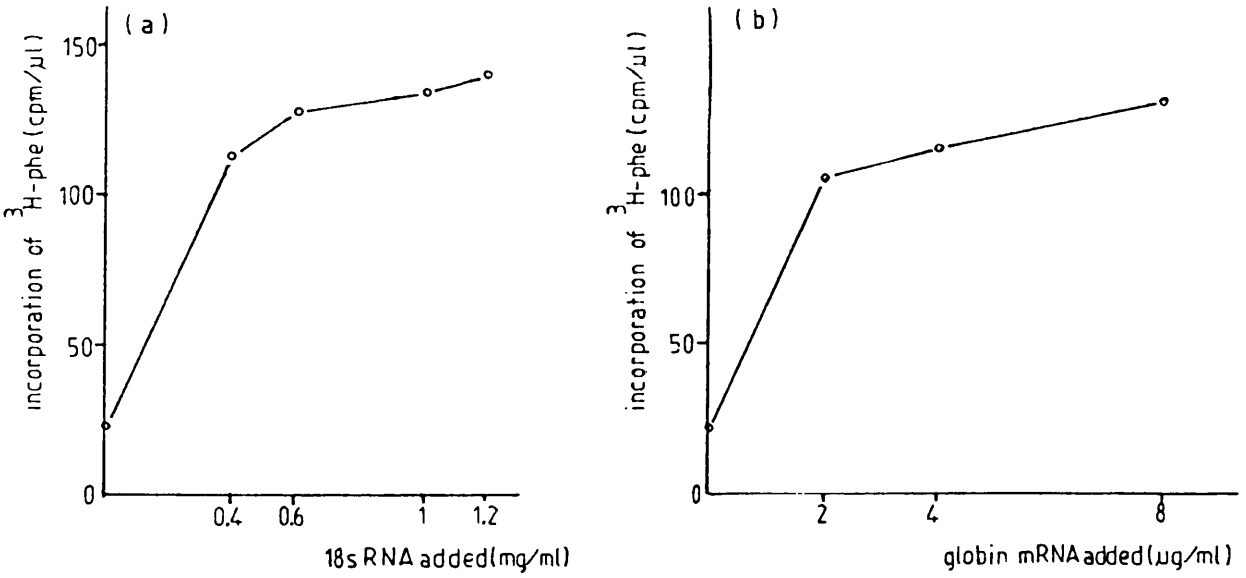


Fig. 3.7. Translation characteristic of 18S RNA from rat liver (a) and rabbit globin mRNA (b) in wheat germ system, showed the response to increasing quantities of RNA.

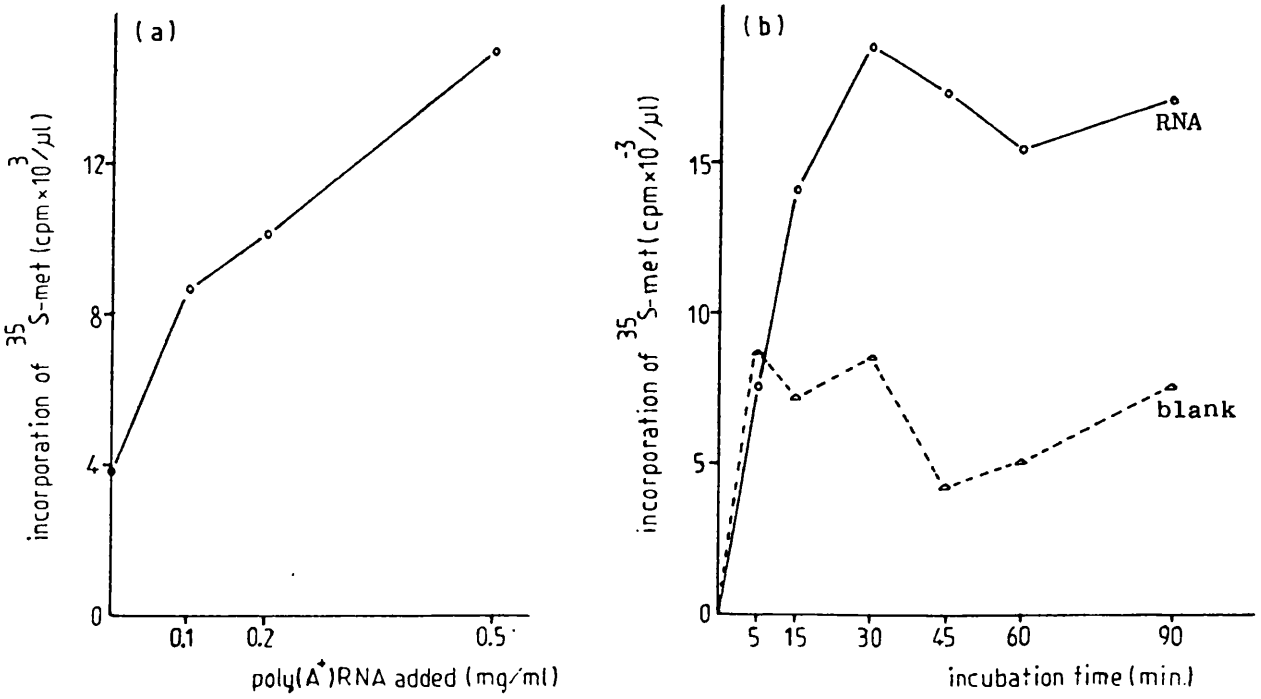


Fig. 3.8. The effect of poly(A)$^{+}$RNA concentration on protein synthetic activity (a) and time-course experiment (b). Poly(A)$^{+}$RNA was extracted from rat liver by using guanidinium thiocyanate procedure. All incubation was carried out for 60 min, at 30°C with ^{35}S-Met in rabbit reticulocyte lysate system.

may not be the most active system. This system had given poor synthesis of large polypeptides and this problem was probably improved by adding polyamine (Roberts et al.,1975). This wheat germ system was proved to work with poly u, which gave the maximum stimulation, about 10 times the level of radioactivity in the blank without addition of exogenous RNA. The poly u was a synthetic polynucleotide and used only the phe to synthesize polyphe, while poly(A)$^+$RNA needs 20 essential amino acids to synthesize a polypeptide chain. By checking with an amino acid analyzer, the wheat germ system which was used to translate the poly(A)$^+$RNA from tissues and cells contained all 19 amino acids and no phe. Therefore, the low efficiency of translation of poly(A)$^+$RNA in wheat germ system may be due to lack of essential materials for initiation and elongation steps and the system may not be in the optimum condition for each type of poly(A)$^+$ RNA (Benveniste et al.,1976;Tse and Taylor,1977).

3.1.2.d. Using guanidinium thiocyanate.

A major problem in isolation of biologically active mRNA from tissues and cells is contamination by RNase. The primary concern has been either the rapid separation of the RNA from nucleases or the rapid inactivation of nucleases. Therefore rapid lysis of the cells and inactivation of the RNases may be achieved by using the method described by Chirgwin et al. (1979). They prepared RNA from tissue rich in RNase (pancrease) by homogenization of the tissue in the presence of a powerful denaturing agent, guanidinium thiocyanate, and 2-mercaptoethanol and found that this method was sufficiently effective to yield undegraded and active cellular RNA.

Rat liver, calf thymus and NS 1 cells were used for isolation of RNA. The recovery of these RNAs was about 25mg per g of wet tissue, or cells and varied by 27%. The average ratio of

Table 3.3. The recovery at each step in preparation of RNA from tissues and cells by the guanidinium thiocyanate procedure.

Source and amount	Yield of total RNA(mg)	Ratio of A_{260}/A_{280} of RNA	% Yield (RNA/g tissue)	Yield of poly(A)$^+$RNA(A_{260}) first cycle	Yield of poly(A)$^+$RNA(A_{260}) second cycle	% Recovery of poly(A)$^+$RNA first cycle	% Recovery of poly(A)$^+$RNA second cycle
Rat liver (10g)	260	1.94	2.60	24.00	7.53	2.31	0.72
Rat liver (12.5g)	186.5	2.01	1.49	15.20	2.96	2.04	0.39
Calf thymus (10g)	264.5	2.10	2.65	21.71	5.56	2.05	0.56
NS1 cells (160×10^6)	27.4	1.96	3.43	4.10	0.62	3.74	0.56

A_{260}/A_{280} was 2.0 ± 0.06. Poly(A)$^+$RNA was purified by two passages over the oligo(dT) cellulose column and the yield of poly(A)$^+$RNA varied from 0.39 to 0.72% of total RNA. At the step of ethanol precipitation of poly(A)$^+$RNA, E.coli tRNA was added as a carrier to improve the recovery (Krystosek et al.,1975). The results for isolation of total RNA and poly(A)$^+$RNA from tissues and cells by this method are summarized in Table 3.3.

As presented in section 3.2.1.c, the efficiency of translation of poly(A)$^+$RNA in wheat germ lysate was very low. Therefore, the conditions in the wheat germ system which were used to translate poly(A)$^+$RNA, were adapted by using 100mM KCl instead of 64mM, 50µM each of 19 essential amino acids instead of 20µM, and incubation at 30°Cinstead of 25°C. The maximum radioactivity incorporation was increased from 10 fold to 15 fold for poly u at the concentration of 20µg in 25µl reaction mixture while the value was unchanged, only 1.5 to 2 fold above a control(without mRNA) for either first or second bound poly(A)$^+$RNA from rat liver and calf thymus at concentrationsof 4-20µg in 25µl reaction mixture. The incorporation was not significantly improved when using a mixture of ^{3}H-Phe and ^{3}H-Leu,the incorporation of radioactivity was 2 to 4 fold higher than a control (no exogenous mRNA).

Rabbit reticulocyte lysate (nuclease treated) was considered for use in checking the activity of isolated poly(A)$^+$RNA from tissues and cells. In the next chapter (section 4.1.2.) this reticulocyte lysate system is shown to be active in translation of mRNA from various sources. The rat liver poly(A)$^+$RNA from two cycles of oligo(dT) cellulose chromatography was found to give a specific stimulation of 4 times the level of radioactivity incorporated in the control (without exogenous mRNA). The incorporation

of ^{35}S-Met into polypeptide chains was linearly dependent on the amount of poly(A)$^+$RNA (Fig.3.8.a.), and incubation time (Fig. 3.8.b.).

3.2.1.e. Using a phenol-chloroform deproteinization procedure.

The isolation of nucleic acid from tissue was done by using the rapid phenol-chloroform deproteinization procedure as described in the method 3.1.1.e. about 0.7g of total nucleic acid was extracted from 12.6g of rat liver (wet weight) and the DNA was separated out by precipitation with 1% streptomycin sulphate pH5. The total RNA yield from rat liver approached 1% of the wet weight. Poly(A)$^+$RNA was purified by two cycles of oligo(dT) cellulose chromatography. The percent yield of poly(A)$^+$RNA was approximately 1% of total RNA and had a A_{260}/A_{280} ratio of 2.25.

The second bound fraction of poly(A)$^+$RNA was active in promoting incorporation of ^{3}H-Leu or ^{35}S-Met, when tested in reticulocyte lysate cell-free systems. The incorporation of ^{3}H-Leu was enhanced with increasing poly(A)$^+$RNA concentration and the maximum stimulation was approximately 7 fold higher than control at a concentration of 0.25µl in 10µl reaction mixture (Fig. 3.9.a). In a time course study, the incorporation of ^{3}H-Leu increased linearly for 10 min and then leveled off (Fig.3.9.b).

This method gave reproducible yields of RNA, took less time in preparation and yielded RNA that was suitable for protein synthesis. The efficiency in translation of this poly(A)$^+$RNA from rat liver was slightly better than poly(A)$^+$RNA that was isolated using guanidinium thiocyanate. These two methods, guanidinium thiocyanate and phenol-chloroform deproteinization procedures, have shown the possibility of isolating poly(A)$^+$RNA from relatively large amounts of tissue and have demonstrated its activity by in vitro translation.

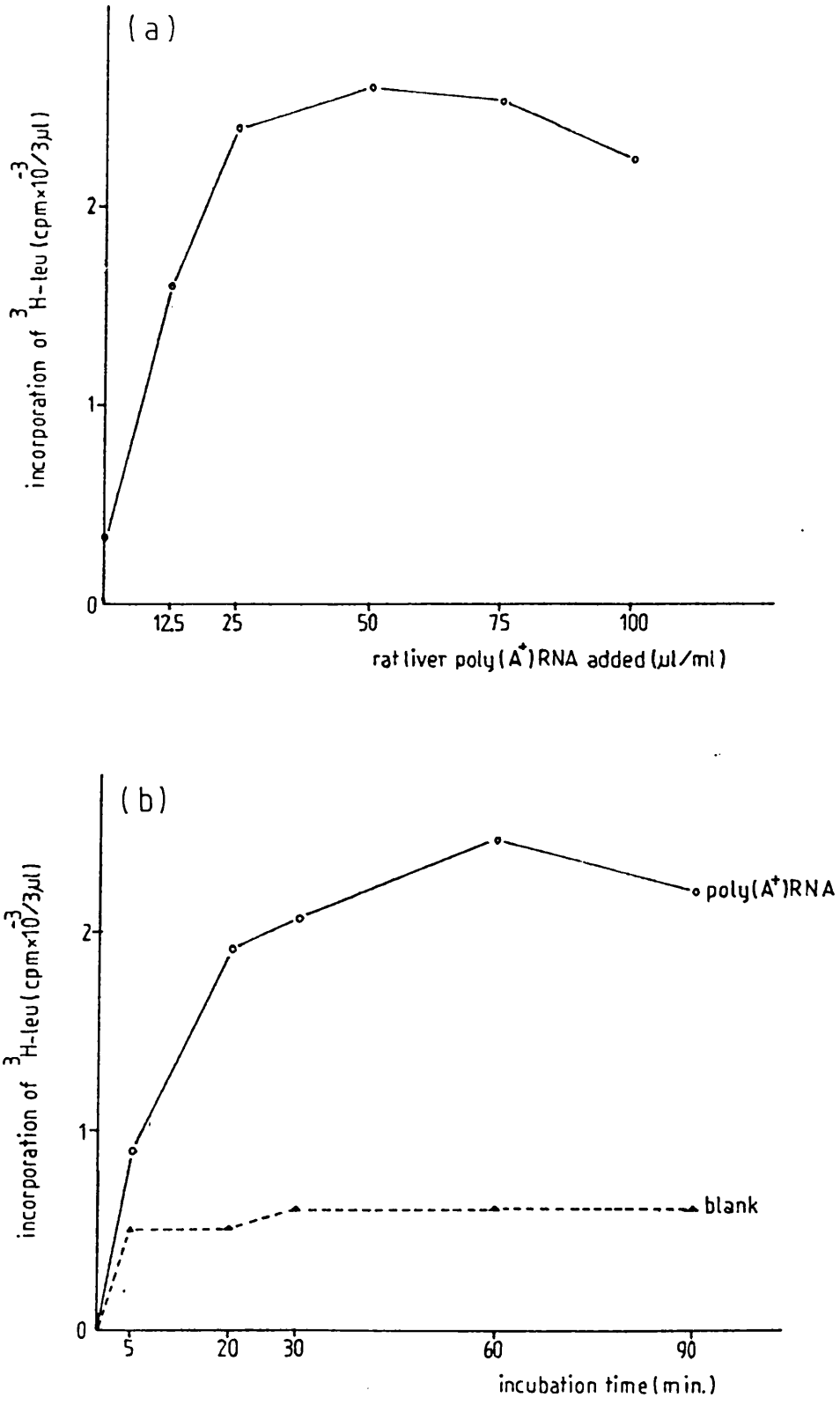


Fig. 3.9. Characteristics of protein synthesis directed by poly(A)$^{+}$RNA from rat liver. Poly(A)$^{+}$RNA was extracted by phenol-chloroform deproteinization procedure and incubated at 30°C for 60 min with ^{3}H-Leu in reticulocyte lysate. a) dose response. b) time-course experiment.

The next step was to attempt to isolate biologically active poly(A)$^+$ RNA from small amounts of cell line material by using rapid phenol-chloroform deproteinization procedure. Rabbit lymphocytes, NS 1, K562 and HMy2 cells were used in this experiment.

Because of the low amount of total nucleic acid isolated from cells, in order to remove DNA from total nucleic acid, RNA was precipitated in 2.5M LiCl (Duguid et al.,1976). The poly(A)$^+$RNA was purified from the LiCl precipitated RNA by oligo(dT) cellulose chromatography.

Rabbit lymphocytes were separated from heparinized blood by using Ficoll-paque centrifugation. About 15-20µg of total nucleic acid were obtained from lymphocytes separated from 1ml of blood. The percent yield of poly(A)$^+$RNA was between 1.5 and 3.59% of total RNA (Table 3.4). The incorporation of ^{35}S-Met was dependent on the amount of poly(A)$^+$RNA and it was about 3 fold greater than mRNA free controlat a concentration of 0.01 A_{260} units in 11µl reaction mixture of reticulocyte lysate (Fig.3.10.a).

For NS 1 cells, the amount of cells used in isolation of poly(A)$^+$RNA was varied and the lowest number of cells that yielded enough amount of active poly(A)$^+$RNA to test in translation was 2.9×10^7 cells. The recovery of total RNA was about 23mg per g of wet weight and varied by 8%. Between 1.67 and 3.43% of total RNA was contained in the polyadenylated fraction after one cycle of oligo(dT) cellulose chromatography. The yield of second bound fraction of poly(A)$^+$RNA from the column was approximately 0.8% of total RNA. The result for the preparation of these RNAs is summarized in Table 3.4. Translation of total RNA, and poly(A)$^+$RNA from either first or second bound fraction in rabbit reticulocyte lysate was characterized by varying RNA concentration as shown in Fig. 3.10.b.

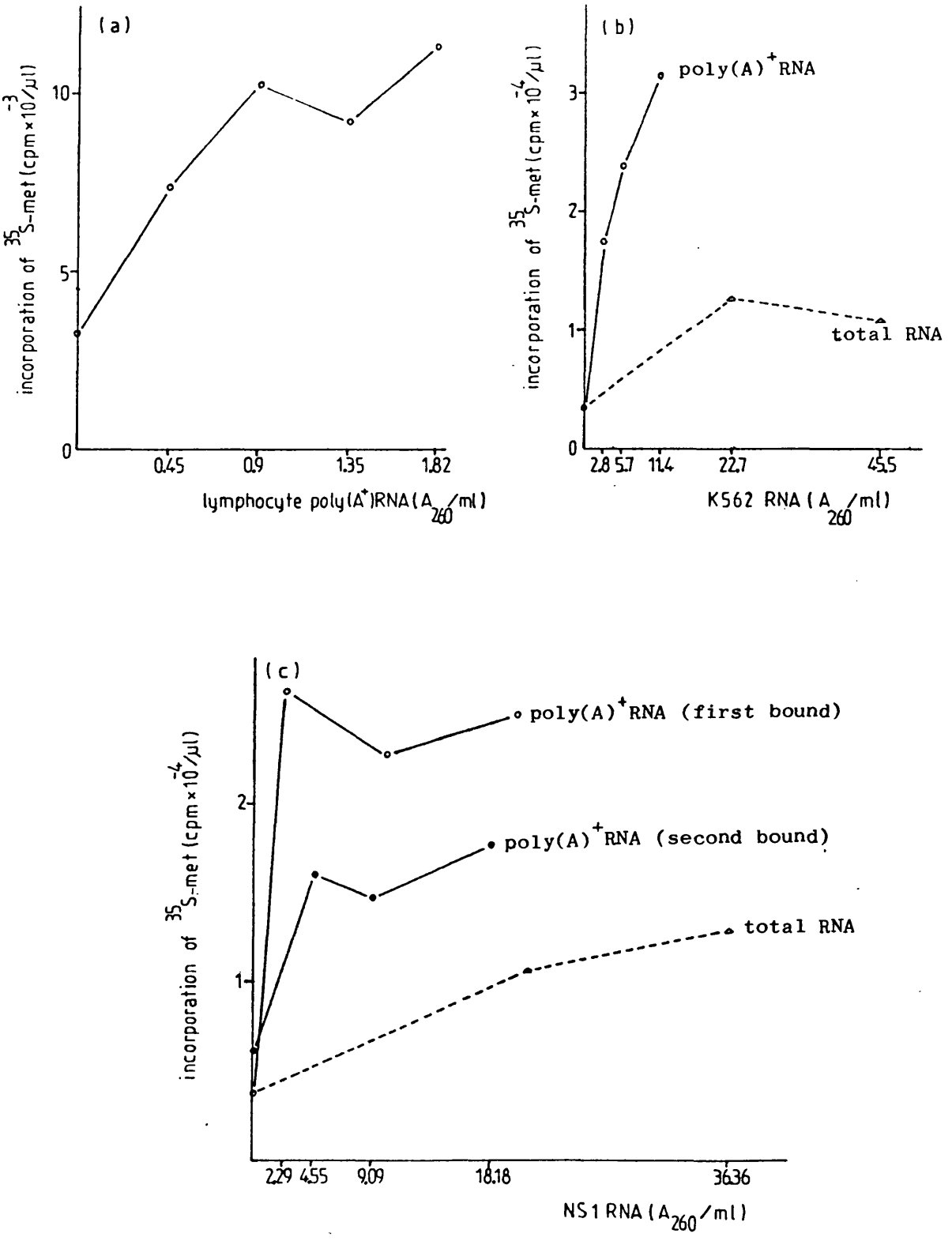


Fig. 3.10. Protein synthesis in response to RNA concentration. Reaction mixtures were incubated at 30°C for 60 min, in rabbit reticulocyte lysate with ^{35}S-Met.

a) poly(A)$^+$RNA isolated from rabbit lymphocytes.

b) RNA isolated from NS1 cells.

c) RNA isolated from K562 cells.

Table 3.4. Purification and recovery of total RNA and poly(A)$^+$RNA from rat liver and cell lines by rapid phenol-chloroform deproteinization procedure.

Source and amount	Yield of total RNA (mg)	% Yield (RNA/g cells)	Yield of poly(A)$^+$RNA (A_{260} units)	% Yield (poly(A)$^+$RNA/ total RNA)	Poly(A)$^+$ RNA from 2nd cycle
Rat liver (12.6g)	127.5	1.01	21.59	4.23	5.04$^+$
Rabbit lymphocyte (80ml blood)	1.65	-	0.237	3.59	-
Rabbit lymphocyte (10ml blood)	0.15	-	0.009	1.50	-
NS1 cells (29 x 10^6)*	3.50	2.33	0.48	3.43	-
NS1 cells (141 x 10^6)*	16.87	2.38	2.23	3.30	0.18$^+$
NS1 cells (66 x 10^6)*	8.40	2.55	0.56	1.67	-
NS1 cells (142 x 10^6)*	14.50	2.04	1.432	2.47	0.09$^+$
K562 cells (100 x 10^6)*	12.75	2.55	1.325	2.59	-
K562 cells (86 x 10^6)*	12.60	2.93	1.66	3.29	-
K562 cells (60 x 10^6)*	8.12	2.71	0.575	1.77	-
K562 cells (46 x 10^6)*	4.0	1.74	0.43	2.68	-
K562 cells (23 x 10^6)*	0.6	0.52	-	-	-
K562 cells (10 x 10^6)*	0.2	0.40	-	-	-
HMy2 cells (28 x 10^6)*	2.21	1.58	0.15	1.69	-

(* 20 x 10^6 cells weigh about 0.1g, + was A_{260} units)

Total RNA at a concentration 0.2 to 0.5 A_{260} units in 11µl reaction mixture gave a radioactivity incorporation of 1.5 to 3.0 fold higher than a control. The incorporation of ^{35}S-Met was increased to as high as 7 fold when using 0.03 A_{260} units of poly(A)$^+$RNA from the first bound fraction. However this activity was not improved by using poly(A)$^+$RNA from the second bound fraction. Its activity was lost and the maximum radioactivity incorporation was only 3 fold above a control (without mRNA) at a concentration as high as 0.2 A_{260} units in 11µl reaction mixture. This may have resulted from using a low amount of poly(A)$^+$RNA from the first bound fraction, therefore, the recovery of the product was low. In addition, the RNA was probably degraded during the step of purification. There was a variation in the activity of RNA from different batches of preparation but the difference was not significant.

For K562 cells, the average recovery of total RNA was quite high, 24.82 ± 4.49mg/g of wet weight cells when prepared from large amounts of cells (at least 6×10^7) but this value was reduced to 4-5mg/g when the number of cells was 10-20 $\times 10^6$ cells. Between 1.77% and 3.29% of poly(A)$^+$RNA was obtained after one cycle of oligo (dT) cellulose chromatography (Table 3.4). The ability of total RNA and poly(A)$^+$RNA to promote the incorporation of ^{35}S-Met into rabbit reticulocyte lysate was compared. As shown in Fig. 3.10.c, the total RNA had lower activity in directing the radioactivity incorporation than poly(A)$^+$RNA. Poly(A)$^+$RNA was relatively active, the incorporation of ^{35}S-Met was approximately 11 fold higher than a control at the concentration of 0.13 A_{260} units in 11µl of reaction mixture, and this incorporation of ^{35}S-Met increased proportionally with RNA concentration.

About 2.8×10^7 cells of HMy2 cell line were used for

isolation of RNA. The recovery of total RNA and poly(A)$^+$RNA was 1.58% and 1.69%, respectively (Table 3.4). The maximum radioactivity incorporation into protein was obtained for RNA concentrations in the total translation reaction mixture of 45 A_{260} units/ml for total RNA and about 5.5 A_{260} units/ml for poly(A)$^+$RNA. Maximum stimulation in both cases was about 3 to 4 fold higher than controls(without mRNA).

3.2.2. Characterization of isolated RNA and synthesized protein by gel electrophoresis.

3.2.2.a. Characterization of isolated RNA from tissues and cells by gel electrophoresis.

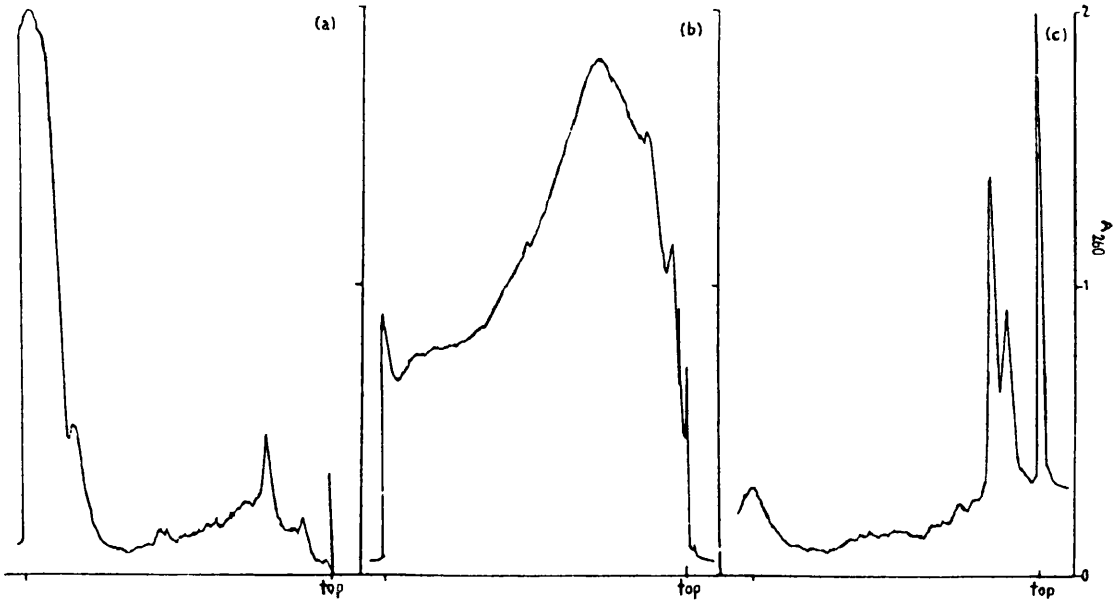
The major aim of this work was to indicate that undegraded RNA can be prepared. RNAs were analysed by electrophoresis under non-denaturing and denaturing conditions in both acrylamide and agarose gel.

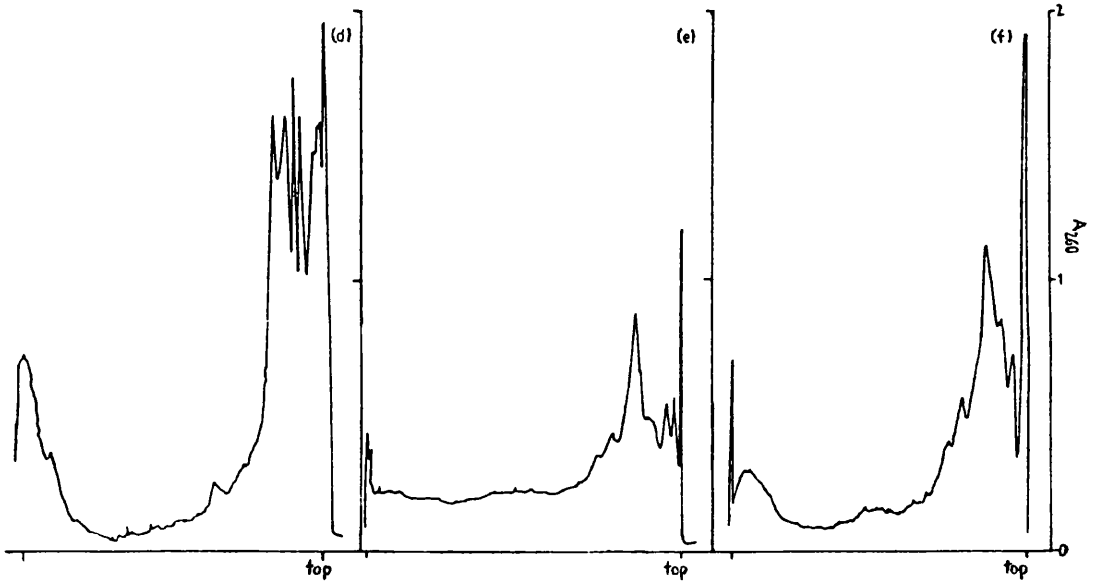
i). Non-denaturing conditions in 4% acrylamide gel.

The RNAs prepared by phenol-chloroform/isoamylalcohol extraction were analysed by electrphoresis on cylindrical gels under non-denaturing conditions in 4% acrylamide (as described in Method 3.1.5.a). After electrophoresis,the gel was washed with water and monitored for RNA by measuring absorbance at 260nm. The absorbance profiles are shown in Fig.3.11.

E.coli tRNA gave one major peak near the bottom of the gel and the Rf value was 0.96 (Fig. 3.11.a). Only one broad peak at an Rf value of 0.29 was shown for poly u (Fig. 3.11.b).

The isolated RNAs were shown to have high M.W. (Fig.3.11. c-g).Two major peaks at an Rf value of 0.12 and 0.17 and one minor peak at an Rf value of 0.95 were obtained for total rat liver RNA. Total calf thymus RNA had 6 major peaks near the top of the gel and one peak at the bottom of the gel and their Rf values were 0.03, 0.05,





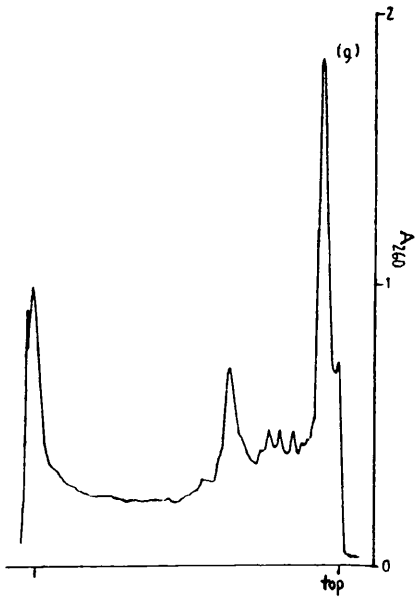


Fig. 3.11. Scan at 260 nm of electrophoresis of RNA on 4% acrylamide gel.

a) tRNA of E.coli

b) poly u

c&d) total rat liver RNA and total calf thymus RNA, respectively. Both samples are prepared by phenol extraction.

e&f) 18S RNA and 28S RNA of rat liver RNA that separated by sucrose gradient ultracentrifugation.

g) 18S polysomal RNA that purified from polysomal RNA of rat liver by sucrose gradient ultracentrifugation.

0.09, 0.12, 0.14, 0.19 and 0.97, respectively.

Total rat liver RNA was further fractionated into 18S RNA and 28S RNA by sucrose density gradient centrifugation. The size distribution of these RNAs on polyacrylamide gel was determined. The absorbance profilesof 18S RNA and 28S RNA were very similar, there was one major peak at Rf value 0.16 and 5 minor peaks at Rf values of 0.02, 0.06-0.07, 0.10-0.11, 0.23 and 0.20. 28S RNA contained higher amounts of RNA at the Rf value of 0.02, than 18S RNA and there was a peak at an Rf value of 0.98 that was not contained in 18S RNA samples(Fig.3.11.e and 3.11.f).

For 18S polysomal RNA, prepared from the polysomal RNA of rat liver by sucrose density gradient centrifugation as in method section 3.1.1.c, 3 major peaks at Rf valuesof 0.06, 0.36 and 0.98,and 4 minor peaks at Rf valuesof 0.15, 0.19, 0.23 and 0.44 (Fig.3.11.g) were obtained.

These results demonstrate the presence of high M.W. cellular RNA when isclated by phenol extraction in the presence of RNase inhibitors, heparin and SDS.

ii). Non-denaturing conditions in 3-8% SDS-PAGE.

The 3-8% polyacrylamide gel was a vertical slab and the separated nucleic acid samples were visualized by their fluorescence under UV light after staining with acridine orange. Calf thymus DNA, poly u and tRNA from wheat germ were used as standards and they showed broad bands at Rf values of 0.17, 0.22 and 1.0, respectively.

Total rat liver RNA, prepared by phenol chloroform deproteinization procedure, gave three major bands at Rf values of 0.15, 0.2 and 0.98. Five bands of Rf values between 0.15 and 0.72 were detected for 18S polysoaml RNA prepared as in the method section 3.1.1.c. Total K562 DNA, separated from total nucleic acid by the

streptomycin precipitation procedure, showed a tailing band near the origin of the gel with an Rf value 0.05. With total K562 RNA, one major band at an Rf value of 0.17 was obtained and no bandscould be detected for K562 poly(A)$^+$RNA, which was probably a result of the low amount of RNA loaded. Degradation was detected in lymphocyte poly(A)$^+$RNA after keeping at -20^{o}C for more than 6 months. One broad band at the Rf value of 0.8 was shown.

The RNAs prepared by phenol extraction showed no degradation products by gel electrophoresis except after prolonged keeping at -20^{o}C. This gel electrophoresis failed to separate high M.W. nucleic acids, even after increasing the electrophoresis time. The DNA and RNA were separated and located at the same position and this may be related to the pore size of the gel. Therefore, agarose gel was used to separate the RNA.

iii). Radiolabelling of RNA in cells.

The labelled RNA was used for checking the recovery of RNA at each step in isolation and purification of RNA from cells by a phenol-chloroform deproteinization procedure and oligo(dT) cellulose chromatography and for determination of the size of isolated RNA by gel electrophoresis.

The RNA from K562 and HMy2 cells was labelled with ^{3}H-uridine and extracted with phenol-chloroform isoamylalcohol by procedures described in method section 3.1.4. and 3.1.1.e, respectively. About 70-80% of total radioactivity was found in the aqueous phase and only 30-40% of this was recovered in the RNA pellet. The total nucleic acid contained only 1.2% poly(A)$^+$RNA. This result was similar to the result determined by absorbance value at 260nm. These RNAsfrom K562 and HMy2 were tested for translational activity in rabbit reticulocyte lysate and the incorporation of ^{35}S-Met was 2-4 fold greater than mRNA

free control at the concentration of 2-10µg in 11µl reaction mixture.

iv). Non - denaturing conditions in 1.2% agarose minigels.

The separated nucleic acid was detected under UV light after staining with ethidium bromide. Calf thymus DNA and wheat germ tRNA gave a broad band at Rf values of 0.37 and 1.2, respectively. Total K562 DNA and RNA, prepared by phenol-chloroform deproteinization procedure, showed one band at the same Rf value of 0.18. This may result from either contamination by DNA in the RNA fraction or this gel electrophoresis may not be able to separate high M.W. nucleic acids. Total nucleic acid of HMy2 showed one band at Rf value 0.24.

Result of fluorography of isolated labelled K562 RNA and HMy2 RNA demonstrated that K562 RNA had four bands at Rf values of 0.27, 0.45, 0.64 and 1.1 (Fig. 3.12. a. lane 1 and 2) while HMy2 RNA showed two faint bands at Rf valuesabout 0.3 and 1.1 (Fig.3.12.a.lane 3).

v). Denaturing conditions in 1.5% agarose gel in urea.

Electrophoresis of labelled K562 RNA and HMy2 RNA in 1.5% agarose gel in 6M urea pH3.5 gave results similar to those found in the 1.2% agarose minigel. ^{3}H-uridine labelled RNA separated from K562 and HMy2 cells (as described in 3.2.2.a. iii) gave a similar heterogenous distribution with two high M.W. bands at Rf values of 0.18 and 0.26. A fluorogram of these labelled RNAsfrom K562 and HMy2 is shown in Fig.3.12.b.lane 1 and 2, and Fig 3.12.b. lane 3 and 4, respectively.

These results demonstrate that nondegraded RNA can be isolated from both tissues and cells by phenol extraction. However, a large amount of tissue and cells was required and the efficiency of translation in the cell-free system was low and variable. Therefore it may not be practical to use these RNAs to study the effect of ANAs.

Fig. 3.12. Fluorogram of ^{3}H-uridine labelled RNA separated by 1.2% agarose minigel (a) and 1.5% agarose gel in 6M urea pH 3.5 (b).

(a)

a)

lane 1. K562 labelled RNA; 10,000 cpm.

lane 2. K562 labelled RNA; 48,000 cpm.

lane 3. HMy2 labelled RNA; 16,000 cpm.

Exposure: 28 days; -70°C.

(b)

b)

lane 1. K562 labelled RNA; 16,000 cpm.

lane 2. K562 labelled RNA; 48,000 cpm.

lane 3. HMy2 labelled RNA; 15,000 cpm.

lane 4. HMy2 labelled RNA; 32,000 cpm.

Exposure: 15 days; -70°C.

3.2.2.b. Characterization of synthesized protein by gel electrophoresis

The protein synthesized from tissue or cell RNA in the reticulocyte lysate system was further analyzed on 5-15% gradient SDS-PAGE. The M.W. of the proteins was determined from a calibration curve of protein markers (as shown in Fig. 2.1.) by using log %T value.

Poly(A)$^+$RNA from rat liver and cell lines directed the synthesis of several proteins and the protein patterns on fluorograms for these RNAs were different. However, the RNA from cell lines, K562, NS 1 and HMy2 gave similar protein patterns.

Rat liver poly(A)$^+$RNA from the second bound fraction directed the synthesis of proteinsof M.W. between 75K and 12K, including the intense bands at M.W. of 65K (albumin), 45K (the band that also was shown without exogenous mRNA Fig.3.13. lane 4) and 26K (Fig. 3.13. lanes 1 and 2). The protein pattern for lymphocyte poly(A)$^+$RNA was relatively simple (Fig.3.13.lane 3) containing only 4 major bands of protein at M.Ws of 65K, 45K, 29K and 14K.

Protein synthesized in response to poly(A)$^+$RNA from K562 and NS 1 gave a similar pattern on fluorogram of SDS-PAGE (Fig.3.13. lane 5-8 and 9, 12 and 13, respectively). The M.W. of these proteins was between 120K and 20K. By scanning the X-ray film of fluorogram of protein synthesized from K562 and NS 1 at 540 nm,it was found that there was a difference at M.W. between 40K and 30K and the intensity of some proteins was different, such as the proteinsat M.W. of 68K,62K, and 41K (Fig.3.14). The protein synthesized from HMy2 and poly(A)$^+$ RNA (Fig. 3.15 lane6 and 7) gave a similar pattern on SDS-PAGE to K562 synthesized protein (Fig. 3.15 lane 3) but the intensity of some bands was different.

This protein pattern of cell lines was quite different from TMV RNA and globin mRNA. Many proteins were synthesized from

Fig. 3.13. A fluorograph of SDS-PAGE of translation product in rabbit reticulocyte lysate.

lane 1: addition of rat liver poly(A)$^+$RNA from second bound fraction
" 2: " of rat liver poly(A)$^+$RNA from second bound fraction
" 3: " of rabbit lymphocyte poly(A)$^+$RNA
" 4: control without addition of exogenous mRNA
" 5: addition of K562 total RNA
" 6: " of K562 total RNA
" 7: " of K562 poly(A)$^+$RNA
" 8: " of K562 poly(A)$^+$RNA
" 9: " of NS1 poly(A)$^+$RNA
" 10: " of globin mRNA
" 11: " of TMV RNA
" 12: " of NS1 total RNA
" 13: " of NS1 poly(A)$^+$RNA

Exposure: 20,000-124,000 cpm; 10 days; -70°C.

Fig. 3.15. Fluorographic image of labeled protein synthesized in rabbit reticulocyte lysate.

lane 1: control without addition of exogenous mRNA
" 2: addition of control normal IgG (as a standard protein marker)
" 3: " of K562 poly(A)$^+$RNA
" 4: " of TMV RNA
" 5: " of HMy2 total RNA
" 6: " of HMy2 poly(A)$^+$RNA
" 7: " of HMy2 poly(A)$^+$RNA
" 8: " of globin mRNA

Exposure: 32,000-154,000 cpm; 17 days; -70°C

Fig. 3.13

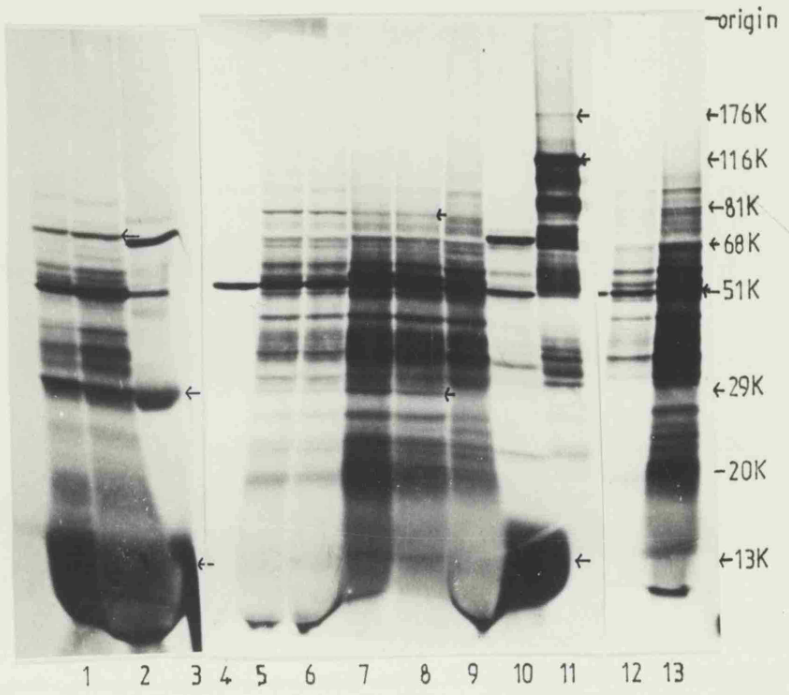
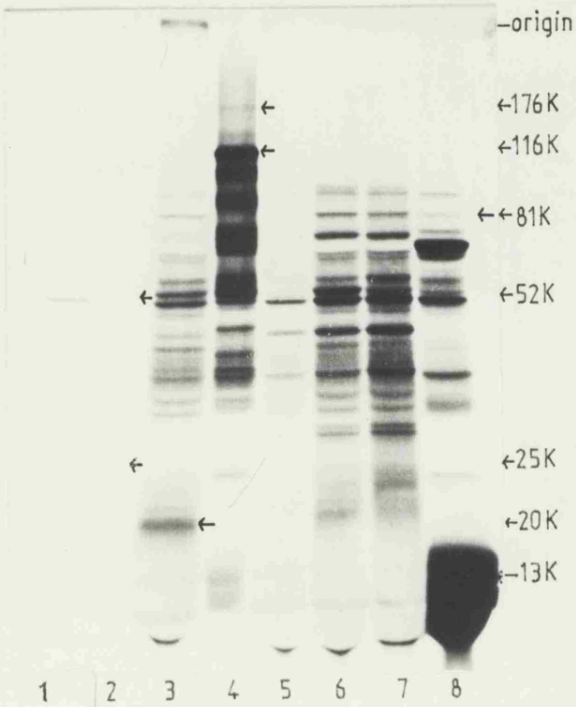


Fig. 3.15



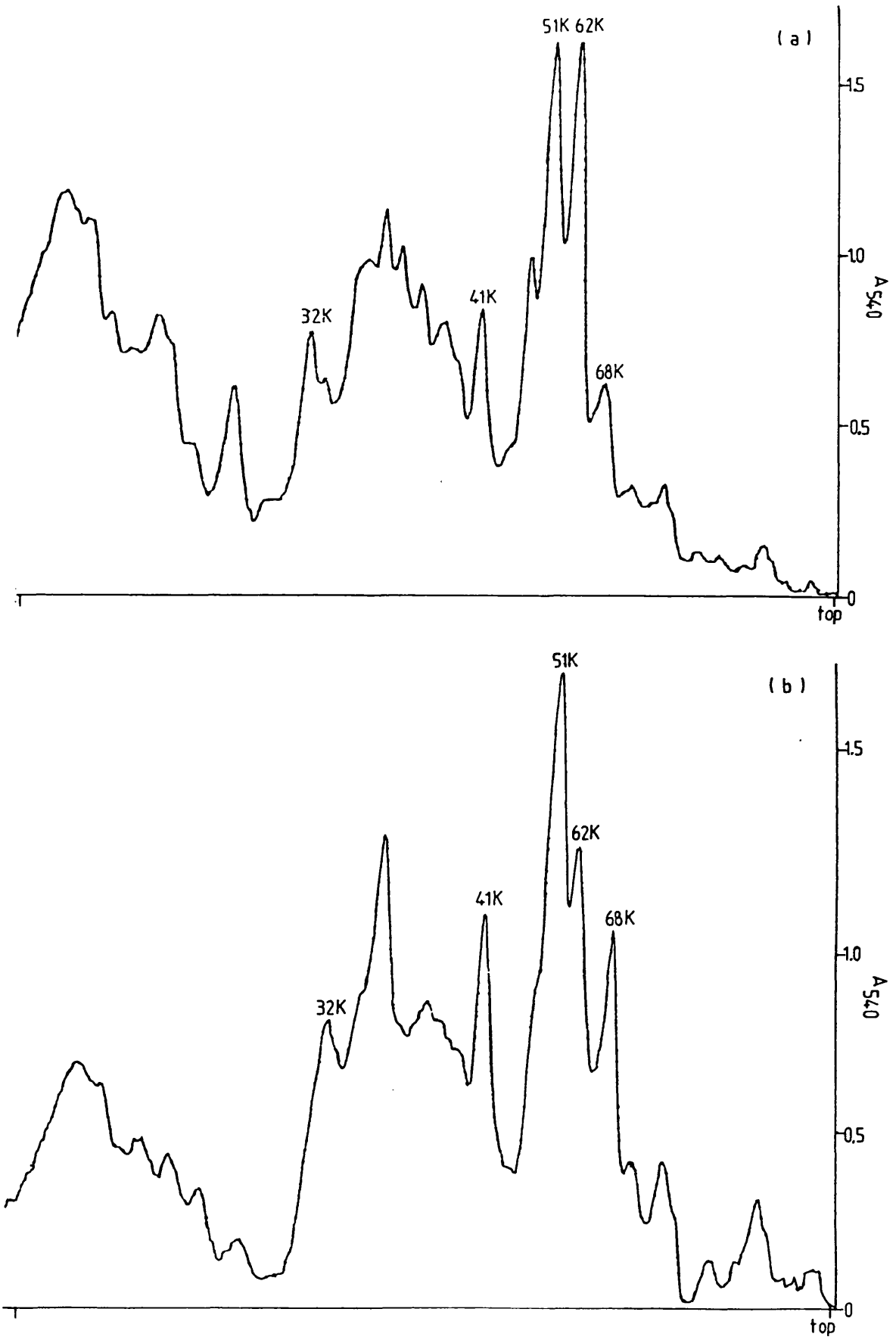


Fig. 3.14. Proteins synthesized from K562 poly(A)$^+$RNA(a) and NS1 poly (A)$^+$RNA(b) were electrophoresed on 5-15% SDS-PAG. A microdensitometer (at 540 nm) trace of an 10 days exposure are shown. Direction of migration was from right to left.

TMV RNA, including proteins at M.W. 176K and 116K (Fig.3.13, lane 11 and Fig. 3.15. lane 4). A major product at M.W. of 13K (globin) and several components at M.W. of 50- 90K, 35-40K and 23K were shown for proteins synthesized from globin mRNA (Fig. 3.13. lane 10 and Fig. 3.15. lane 8).

3.3. Conclusion.

The major aim of this section of work was to isolate undegraded and active mRNA from a small number of cells. A number of different methods for the isolation of active mRNA from a wide variety of sources have appeared. In each case the primary concern was to overcome two main obstacles, damage of mRNA by strong shearing forces and hydrolysis by nucleases (RNase). Enzymic hydrolysis causes the greatest damage to RNA, so that either rapid separation of RNA from nuclease or rapid inactivation of nuclease was required. Further purification of RNA is frequently achieved by affinity chromatography or by sucrose gradient sedimentation. Reviews of these procedures in isolation and purification of RNA, including their developments, can be found in many texts, such as Brawerman 1974, Stewart and Letham,1977; Taylor,1979 and Maniatis et al.,1982.

Many problems were encountered in our attempts to isolate active RNA from small amounts of tissue or cells. Modifications of published procedures which were used with rat liver, high RNase containing tissue or cells have been described for isolation of total RNA from tissues and cell lines (Kahn et al.,1981; Mechler and Rabbitts, 1981;Taylor and Schimke,1973; Chirgwin et al.,1979 and Noyes et al.,1979 or method a-e as described in method section 3.1.1.). The characterization of RNAs was usually based on their efficiencies in translation in cell-free systems (wheat germ lysate and rabbit reticulocyte lysate) and their purities and lack of undegraded products by gel electrophoresis

analysis (polyacrylamide and agarose gel). From these 5 methods for RNA isolation data were obtained relating to the recovery of total RNA and poly(A)$^+$RNA and the activity in protein synthesis in cell-free systems. Only three of these methods (c,d and e) produced an active RNA which translated at a significant rate and gave reliable results.

A large amount of starting material was required to produce enough poly(A)$^+$RNA for study in cell-free translation sytems. Therefore, tissues such as rat liver, rat muscle, and calf thymus were firstly used to investigate an effective procedure for isolation of highly active RNA.

Generally in procedures used here, tissues or cells were gently disrupted in a buffer solution containing dissociating agents or nonionic detergents (such as sodium deoxycholate, SDS, triton X-100, sodium-N-lauroylsarcosine, antifoam A), RNase inhibitors (sodium heparin, DTT and iodoacetate) or potent denature agents (guanidine hydrochloric acid and guanidinium thiocyanate). Apparently,this combination of reagents reduced endogenous RNase levels whereas exogenous nuclease can be minimized by use of sterile gloves and autoclaved glassware and solutions. This homogenate may be further fractionated for polysomes by sucrose gradient centrifugation (method b and c) and isolation of nucleic acid was achieved by extraction of either cell homogenatesor polysomeswith phenol at various pH and ionic strength values and in the presence of chloroform and isoamylalcohol (method b and e). Total RNAs were precipitated from aqueous phase using their properties of having different solubility in salt and certain solvents. RNA of high M.W. was insoluble in 4M NaCl, 2.5M LiCl or 0.2M Na(OAc) or NH_4(OAc) with ethanol (Duguid et al.,1976; Osterberg et al.,1975) whereas tRNA and DNA were soluble. The RNA precipitate can be washed with 3M Na(OAc) pH5 to separate

glycogen, other polysaccharides, DNA and small RNA which may still be left in the preparation at this stage (Palmiter, 1974; Kahn et al.,1981). Total RNA was further purified to 18S RNA,from rat liver,by sucrose gradient centrifugation (Taylor and Schimke,1973; Bantle et al.,1976). and to poly(A)$^+$ RNA by oligo(dT) cellulose chromatography (Aviv and Leder,1972). Salt or detergent contamination was mostly removed from these RNA samples beforeuse in cell-free protein synthesizing systems by passing through the Sephadex G-25 minisystem (Osterburg et al.,1975).

The data presented here indicatesthe considerable variation in yield of total RNA from tissue or cells with different procedures, for example only 0.01% of total RNA was recoveredusinga guanidine-HCl procedure and an improved yield (more than 2%) was achieved with guanidinium-thiocynanate and rapid phenol-chloroform deproteinization procedures (Table 3.1, 3.3 and 3.4 respectively). The reduced activity and low yield of isolated RNA in guanidine-HCl proceduresand polysome isolation may be related to nuclease activity, preparative time and the number of manipulations such as several precipitations and ultra-centrifugations. Lomedico and Saunders (1976) suggested that the long time required and many manipulations involved in polysome isolation created many opportunities for RNA degradation. Moreover, there was a loss of RNA. which did not engage on ribosomes. Total nucleic acid extraction circumvented these problems. Thus, the guanidinium thiocyanate procedure and rapid phenol-chloroform deproteinization procedure seemed to control the degradation of RNA and resulted in active translatable RNA (Fig. 3.8-3.10). Production of undegraded biologically active RNA by these two methods was also demonstrated by Lomedico and Saunders 1976, Noyes et al.1979, Chirgwin et al. 1979 and Allen et al.1982. Most of total RNA samples had A_{260}/A_{280} ratiosof approximately 2.0 indicative of

highly purified RNA (Osterburg et al.,1975; Kahn et all981; Manistis et al.,1982).

Size fractionation on sucrose density gradients and chromatography on oligo(dT) cellulose were performed in purification of eukaryotic mRNA. Following sucrose gradient centrifugation of polysomal RNA from rat liver, 2 major peaks (28S and 18S) were observed as shown in Fig 3.5. This result was also demonstrated by Taylor and Schimke(1973). The translation of 18S RNA in wheat germ lysate, under conditions which were optimal for poly u, indicated the maximum radioactivity incorporation of about 4 fold over free mRNA control (Fig 3.7,a) whereas the maximum incorporation of ^{3}H-Phe directed by globin mRNA and poly u was approximately 3.5 and 10 fold, respectively (Fig. 3.7.b). Therefore,the optimum conditions with wheat germ lysate for poly u were not suitable for rat liver 18S RNA and globin mRNA. Various reports by other laboratories found that mRNA species might not all have the same assay requirement and that minor changes in assay conditions, especially K^{+} and Mg^{2+} ion concentration could affect the translational efficiency in the wheat germ lysate cell-free system (Marcu and Dudock, 1974; Benveniste et al.,1976;Tse and Taylor,1977). Increasing the concentration of K^{+} (from 64mM to 100mM) and amino acids (from 20µM to 50µM) in the translation of poly(A)$^{+}$RNA from rat liver and calf thymus (isolated by guanidinium thiocyanate) did not improve the translation efficiency. The rabbit reticulocyte lysate cell-free system was utilized in the study of translation activity of isolated RNA instead of wheat germ lysate. This cell-free system was very active in translation of various mRNAs as shown in chapter 4, section 4.1.2.

Selective purification of mRNA by the sucrose gradient

centrifugatiom method was time consuming, used many centrifugations and obtained only certain size classes of mRNA. Therefore,the simple and most commonly used method of oligo(dT) cellulose chromatography was applied in the purification of eukaryotic mRNA and it appeared possible to obtain poly(A)$^+$RNA free of contaminating rRNA (Aviv and Leder,1972). The yield of poly(A)$^+$RNA was 0.6-4% and 0.4-0.7% of total RNA after one cycle and two cycles of oligo(dT) cellulose chromatography, respectively. The variation in the level of poly(A)$^+$ RNA might result from the efficiency in isolation of undegraded total RNA (Brawerman,1974) or the source of tissue or cells, since typical mammalian cells contain mRNA between 1% and 5% of the total cellular RNA (Maniatis et al.,1982). The translational activity of poly(A)$^+$RNA which was purified by one cycle of oligo(dT) cellulose chromatography was increased more than 50 times compared with that of total RNA. The purity or translation activity of poly(A)$^+$RNA was not enhanced by a further purification on a second column of oligo(dT) cellulose as also shown by Krystosek et al(1975) so that only one cycle of oligo(dT) cellulose chromatography was performed in purification of poly(A)$^+$RNA from cells. The rapid phenol-chloroform deproteinization had several advantages over other methods which included a higher yield of RNA, an easy, rapid and reproducible method and it provided biologically active poly(A)$^+$RNA. This method was used to isolate poly(A)$^+$RNA from various cells lines and the results are presented in Table 3.4. The poly(A)$^+$RNA from these cell lines (rabbit lymphocyte, NS 1, K562 and HMy2 cells) gave more than a 3 fold increase in radioactivity incorporation over the control in rabbit reticulocyte lysate cell-free system (Fig. 3.10).

By analysis of RNA on gel electrophoresis (both PAG and agarose gel), unlabelled RNA was most commonly detected by absorbances

at 260nm (Fig. 3.11) and by staining with acridine orange or ethidium bromide whereas labelled RNA was detected by fluorography (Fig. 3.12). The results confirmed that undegraded total RNA can be prepared by either the phenol-chloroform deproteinization procedure or purification by sucrose gradient centrifugation. These RNAs gave bands at high M.W, with Rf values less than 0.3, after analysis on gel electrophoresis in either nondenaturing or denaturing conditions. The results presented are similar to Chirgwin et al(1979) and Krystosek et al(1975). They demonstrated the presence of high M.W. RNA at the position of 28S and 18S rRNA (or 9S RNA for globin mRNA) in gels with undegraded RNA whereas only one peak at low M.W. (near tRNA position) was detected after separation of degraded RNA by gel electrophoresis. Therefore the results of RNA gel electrophoresis demonstrated the lack of degradation of the RNA product when isolated by phenol-chloroform extraction.

These RNAs were effective in directing the synthesis of many proteins. The incorporation of radioactivity was dependent on both RNA concentration and incubation time (Fig. 3.8-3.10). Fluorographic analysis of the translation products, resolved by one-dimensional SDS-PAGE, confirmed that the phenol-chloroform and guanidinium thiocyanate extraction yielded RNA suitable for protein synthesis in rabbit reticulocyte lysate cell-free systems. A number of proteins were synthesized in response to poly(A)$^+$RNA from rat liver, K562, NS 1 and HMy2 cells (Fig.3.13 and 3.15). The protein profile of synthesized rat liver protein (Fig. 3.13 lane 1 and 2) was the same as shown by Pelham and Jackson (1976), Chirgwin et al. (1979); Moffett and Webb (1981) and Barth et al. (1982). Many polypeptides (M.W. between 75K and 12K) of various abundances were shown including a band that corresponds to albumin (M.W.68K).

The protein profile was similar for translation products of poly(A)$^+$ RNA from cell lines(K562, NS 1, and HMy2 cells), as shown in Fig.3.15 lane 5-9, 12 and 13 and Fig.3.15 lane 3 and 5-7. The M.W. of protein was between 120K and 20K with difference in intensity of some bands between these proteins from different typesof cell (Fig.314).

The results presented here demonstrate that active poly(A)$^+$ RNA can be purified from both tissue and cells and that these RNAs also show a high efficiency to direct protein synthesis in the rabbit reticulocyte lysate cell-free system. Since a large amount of cells was required to obtain enough poly(A)$^+$RNA for further study, and the variation of results may be found due to translation of these poly(A)$^+$RNA under different conditions in the cell-free system, it was difficult to utilize these poly(A)$^+$RNA routinely to investigate the effects of ANAson the mRNA production in cell linesand consequent effects on protein synthesis.

Chapter Four Protein Synthesis in Cell-free Systems.

4.1. Results

4.1.1. Protein synthesis in wheat germ lysate cell-free system.

4.1.1.a. Conditions for polyphe synthesis directed by poly u.

4.1.1.b. Analysis of polyphe by SDS-PAGE and sucrose gradient centrifugation.

4.1.1.c. Translation of TMV RNA.

4.1.2. Protein synthesis in rabbit reticulocyte lysate cell-free system.

4.1.2.a. Translation of TMV RNA.

4.1.2.b. Translation of globin mRNA.

4.1.2.c. Translation of poly(A)$^+$RNA from rat liver, K562 and NS1 cells.

4.1.2.d. Translation of poly u.

4.2. Conclusion

4. Protein Synthesis in Cell-free Systems.

The optimal conditions for translation of various RNAs in cell-free systems were investigated and selected for use in studying the effect of ANAson protein synthesis in chapter 5.

4.1. Results.

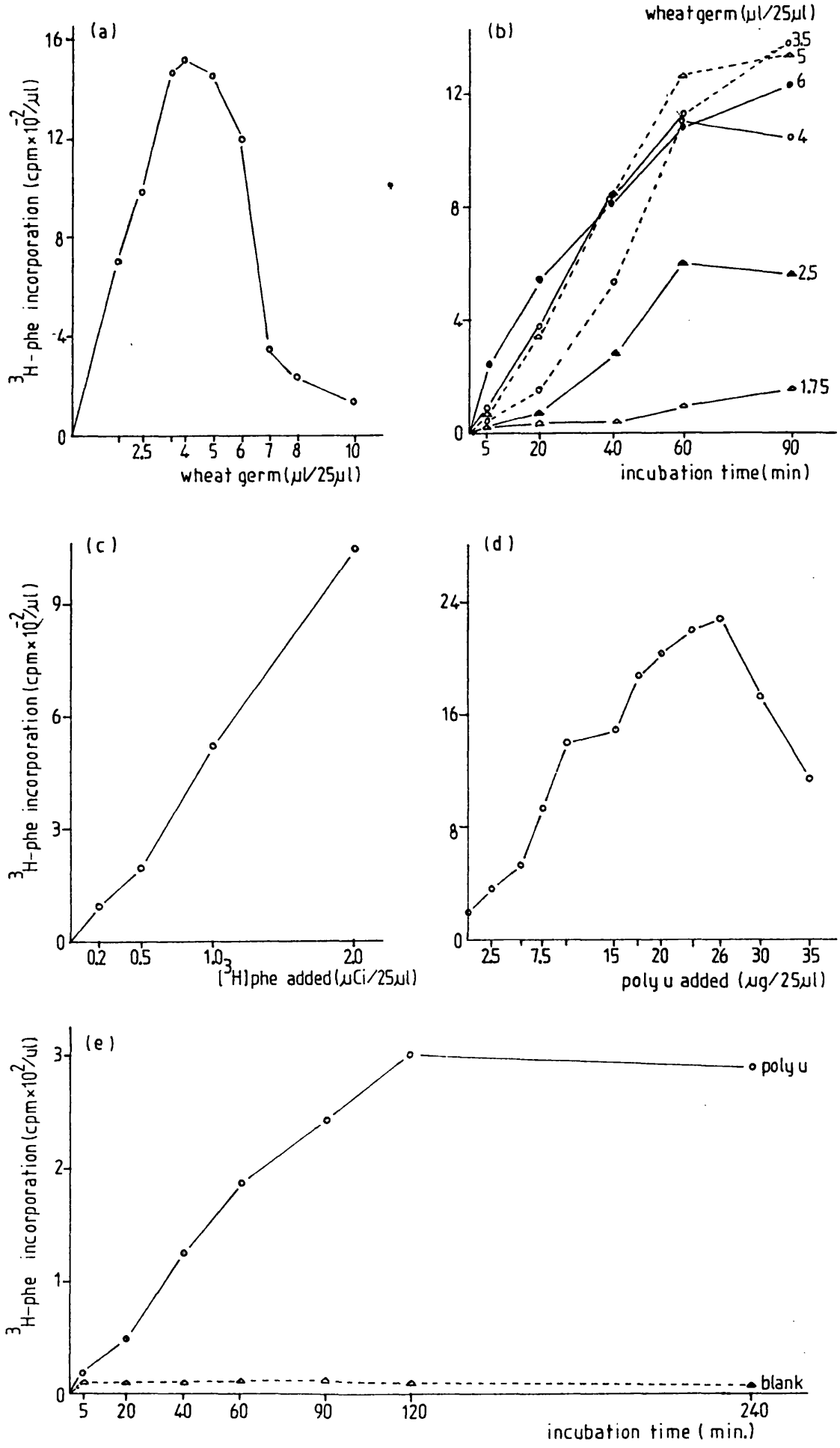
4.1.1. Protein synthesis in wheat germ lysate cell-free system.

4.1.1.a. Conditions for polyphe synthesis directed by poly u.

An artificial messenger, poly u, was used for initial optimization for translation in wheat germ lysate cell-free system. The final concentration of the components used in this cell-free system was described in method section 2.2.6.

The effect on translation of varying the concentration of wheat germ lysate was examined and it was found that the highest activity for poly u translation was given at amounts of wheat germ lysate between 3.5µl and 5µl (or 189µg and 270µg of lysate protein) in 25µl reaction mixture (Fig. 4.1.a. and 4.1.b.). The radioactivity incorporation directed by poly u was dependent on the concentration of added ^{3}H-Phe and incubation time (Fig.4.1.c. and 4.1.e., respectively). After an initial lag of 5 to 10 min, the incorporation of ^{3}H-Phe in response to poly u increased linearly for 120 min and terminated after 240 min (Fig. 4.1.e.). Concentrations of poly u between 0.80 and 1.05mg/ml gave the highest radioactivity incorporation as shown in Fig. 4.1.d.

Polyamines, in particular spermine and spermidine, have been shown to have a stimulatory effect on protein synthesis both in prokaryotic and eukaryotic cell-free systems (Hunter et al.,1977; Igarashi et al.,1979; Abraham et al.,1979). Abraham et al.(1979) showed that the rate of synthesis of polyphe was significally enhanced when Mg(OAc) was partially replaced by spermidine (1.5mM


(a)
^{3}H-phe incorporation (cpm×10^{-2}/µl)
wheat germ (µl/25µl)
(b)
wheat germ (µl/25µl)
3.5
5
6
4
2.5
1.75
incubation time (min)
(c)
^{3}H-phe incorporation (cpm×10^{-2}/µl)
[^{3}H]phe added (µCi/25µl)
(d)
poly u added (µg/25µl)
(e)
^{3}H-phe incorporation (cpm×10^2/ul)
poly u
blank
incubation time (min.)

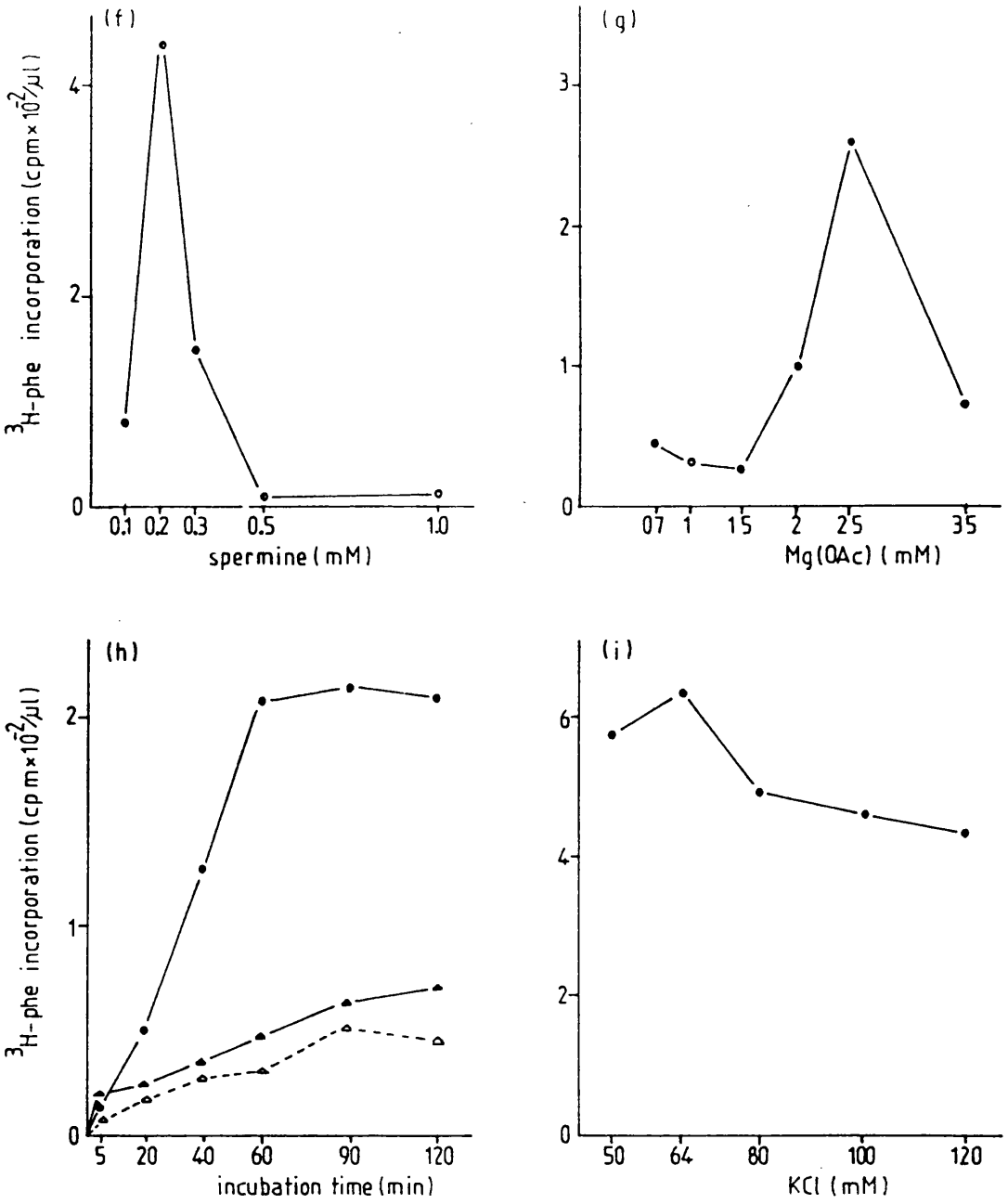


Fig. 4.1. Characteristics of polyphe synthesis directed by poly u in wheat germ cell-free system.

a) effect of wheat germ lysate concentration on translation of poly u

b) effect of wheat germ lysate concentration and incubation time on translation of poly u.

c) polyphe synthesis at various concentrations of ^{3}H-Phe.

d) response to increasing amounts of poly u.

e) kinetics of reaction without added poly u (▲----▲) and with 800 µg/ml of poly u (●——●).

f) effect of spermine HCl concentration on translation of poly u

g) effect of Mg(OAc) concentration on translation of poly u.

h) effect of spermine HCl and Mg(OAc) concentration on translation of poly u; 0.2mM spermine HCl and 2.5mM Mg(OAc) (●——●); 3.5mM Mg(OAc) (▲——▲); and 2.5mM Mg(OAc) (●----●).

i) effect of KCl concentration on translation of poly u.

The reaction mixture (except b,e, and h) was incubated at 25°C for 90 min and radioactivity was determined in 10 µl of reaction mixture. All points were the average of duplicates.

Mg(OAc) and 0.66mM spermidine). A similar result was found in our experiment with different concentration of spermidine and Mg(OAc). For translation of poly u in wheat germ lysate which contained both spermidine and Mg(OAc), the optimum concentration of spermidine hydrochloride in the presence of 3.5mM Mg(OAc) was 0.2mM (Fig. 4.1.f). The addition of optimum concentration of spermidine hydrochloride resulted in a lowering in the optimal Mg(OAc) concentration from 3.5mM to 2.5mM as shown in Fig.4.1.g. This result was also shown by Hunter et al. (1977). The concentration of Mg(OAc) was reduced from 3.5mM to 1.5mM when adding optimum spermidine concentration (0.8mM) in translation of TMV RNA in wheat germ lysate cell-free system.

The stimulatory effect of spermidine on poly u translation is clearly shown in Fig.4.1.h. At the optimum spermidine hydrochloride concentration (0.2mM) in the reaction mixture containing 2.5mM Mg(OAc), there was approximately a three fold stimulation of incorporation over that of the reaction mixture containing the optimum Mg(OAc) concentration (3.5mM) or of reaction mixture containing 2.5mM Mg(OAc). For further experiments, we routinely used 2.5mM Mg(OAc) and 0.2mM spermidine hydrochloride.

The optimal concentration of KCl for polyphe synthesis was found to be 64mM (Fig.4.1.i). From these experiments, we found the optimum conditions in wheat germ cell-free system, which was very active in translation of poly u. This optimum condition in wheat germ lysate cell-free systems was used to test the translation activity of total RNA or poly(A)$^+$RNA isolated from tissue and cell lines as previously described in Chapter 3.

Endogenous incorporation (in a control without poly u) was very low in wheat germ lysate cell-free systems; it was less than 5%.

4.1.1.b. Analysis of polyphe by SDS-PAGE and sucrose gradient centrifugation.

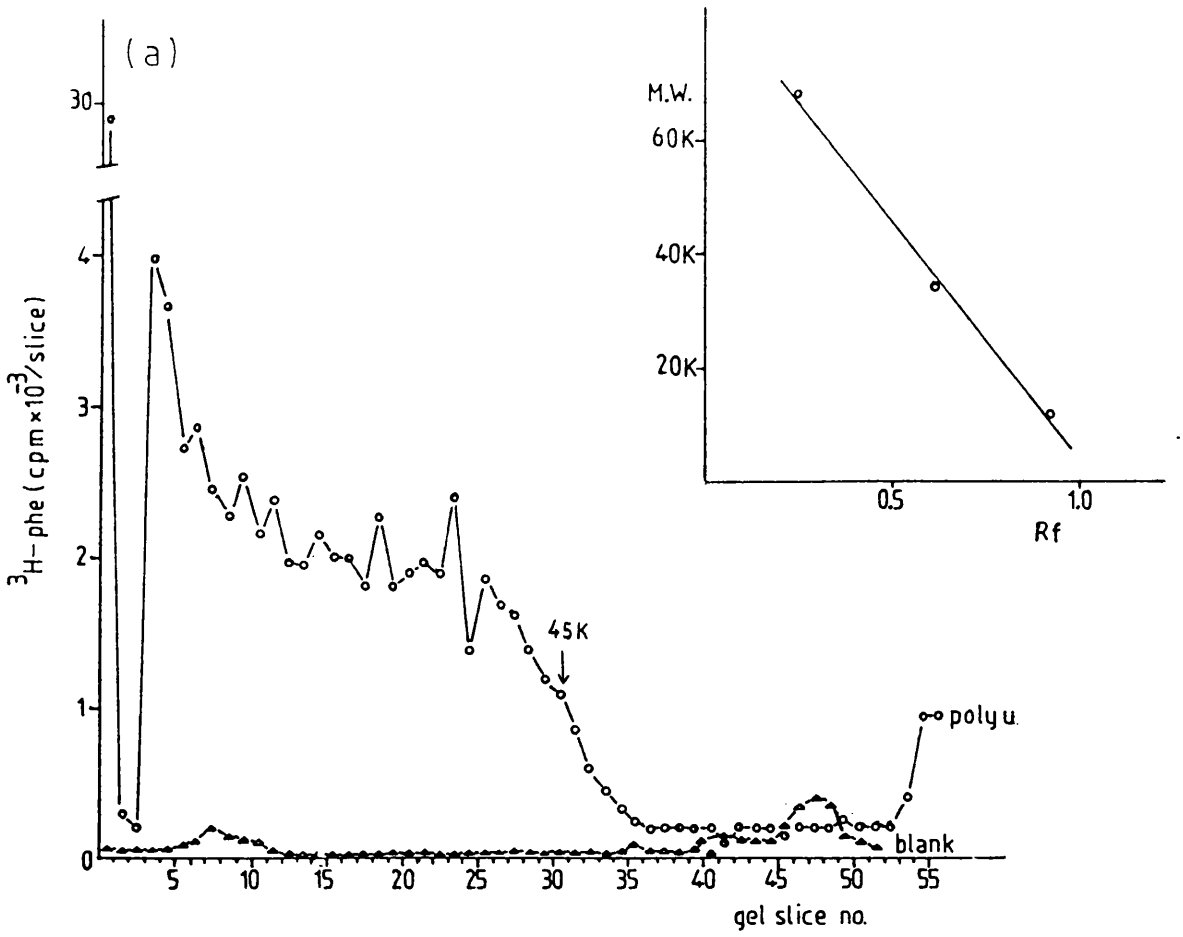
The translation product in vitro of poly u in wheat germ lysate was further analysed on SDS-PAGE/fluorography and sucrose gradient centrifugation. The results revealed a high yield of a large polypeptide.

Cylindrical 7.5% acrylamide gels were used to separate the trichloroacetic acid-precipitable product from translation of poly u and radioactivity was determined by gel slicing as described in method section 2.2.7.a. TheM.W. of separated proteins on gel was determined from a calibration curve by using its Rf value. As shown in Fig.4.2.a, a broad radioactivity peak that migrated in the M.W. range between 90K and 45K was obtained from translation of poly u in wheat germ lysate. The broadness of the radioactivity peak might be due to the presence of various sizes of synthesized polyphe.

The size of the polyphe synthesized in wheat germ lysate was also determined by sucrose gradient centrifugation. (The method as described by Krystosek et al., 1975). 25µl of translation product was layered on 4.8ml of 10-30% sucrose gradient in a buffer containing 0.01M KCl, 0.0015M $MgCl_2$, 0.001M DTT, and 0.01M Tris-HCl pH7.35 and centrifuged at 39,000 rpm ($200,000 \times g_{max}$) for 2 hr, at 20°C, in a Beckman SW 50.1 rotor. The radioactivity was determined in 30µl of each fraction (8 drop fractions of the gradient). A sharp radioactivity peak sedimented at the fraction near the high density of the sucrose (Fig.4.2.b). These results showed that wheat germ lysate cell-free systemshad a high efficiency to synthesize a large polyphe directed by poly u.

4.1.1.c. Translation of TMV RNA.

A complete translation kit from BRL.Ltd. was used in this experiment and the details of this method were already described in



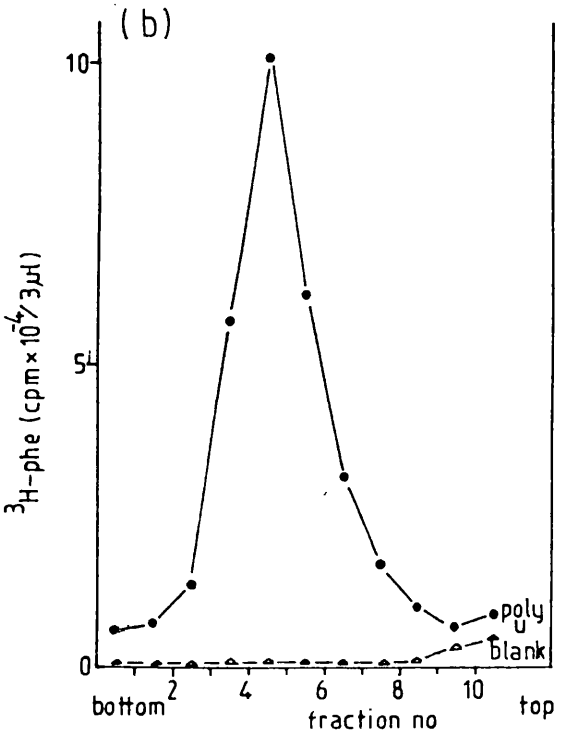


Fig 4.2. a) SDS-PAGE of in vitro synthesized polypeptide in wheat germ lysate. Polyphe was synthesized at 25°C for 90 min and was electrophoresed on 7.5% acrylamide gel. The radioactivity content was determined in each 1.5 mm slice by liquid scintillation counting after solubilizing the gel in hyamine hydroxide and soluene 350. The radioactivity in the gel is illustrated; control (▵——▵) and added poly u(◦——◦). The position of 45K polypeptide is indicated. The calibation curve of standard protein was determined by separation of standard proteins in the parallel gel.

b) Sedimentation behavior of translated product in wheat germ lysate using poly u as template. Sucrose gradient(10-30%) centrifugation and measurement of radioactivity were carried out as described in section 4.1.1.b. (•——•) added poly u and (▵——▵) without poly u.

method section 2.2.6. TMV RNA was translated in a reaction mixture containing 26.7mM HEPES pH7.4, 103mM K(OAc), 3.07mM Mg(OAc) 80μM spermidine phosphate, 0.33mM DTT, 1.2mM ATP, 0.1mM GTP, 5.5mM creatine phosphate, 400μg/ml creatine kinase, 50μM each of 19 amino acids minus methionine, $26A_{260}$ units/ml of wheat germ lysate and 20 - 25 μCi of ^{35}S-Met. The incorporation of radioactivity directed by TMV RNA at concentrations of 5.3μl/ml was approximately 9 fold higher than control (without exogenous mRNA). Fig. 4.3 shows the time-course of ^{35}S-Met incorporation into TCA insoluble material from 1μl reaction mixture. The incorporation of ^{35}S-Met increased almost linearly with incubation time for the first 60 min and then slightly changed until 120 min of incubation.

The synthesized polypeptides directed by TMV RNA in wheat germ lysate were analyzed on a slab gel (5-15% gradient acrylamide) and followed by fluorography as described in method section 2.2.7.a. A fluorogram of synthesized TMV protein revealed the presence of a large number of polypeptides with M.W. between 10K and 120K (Fig. 4.5 b. lane 5 and 6). A large proportion of smaller M.W. protein products were seen and there was no TMV protein at M.W. of 176K when compared to translation product of TMV RNA in rabbit reticulocyte lysate cell-free systems (Fig. 4.5.b. lane 2-4). This result agrees with the finding of low M.W. productsin translation of TMV RNA in wheat germ lysate by Hunter et al(1977). In wheat germ system, a certain amount of nucleolytic cleavage of mRNA occurs, and could account, at least in part, for the synthesis of incomplete chains(Scheele and Blackburn,1979; Hunter et al.,1977). In the presence of polyamine, the nucleolytic cleavage of mRNA was decreased (Abraham et al.,1979) elongation rather than initiation was increased (Hunter et al.,1977) and the binding of aminoacyl tRNA to ribosomeswas high (Igarashi et al.,1982) leading to increased synthesis of full length product, with a corresponding reduction in the

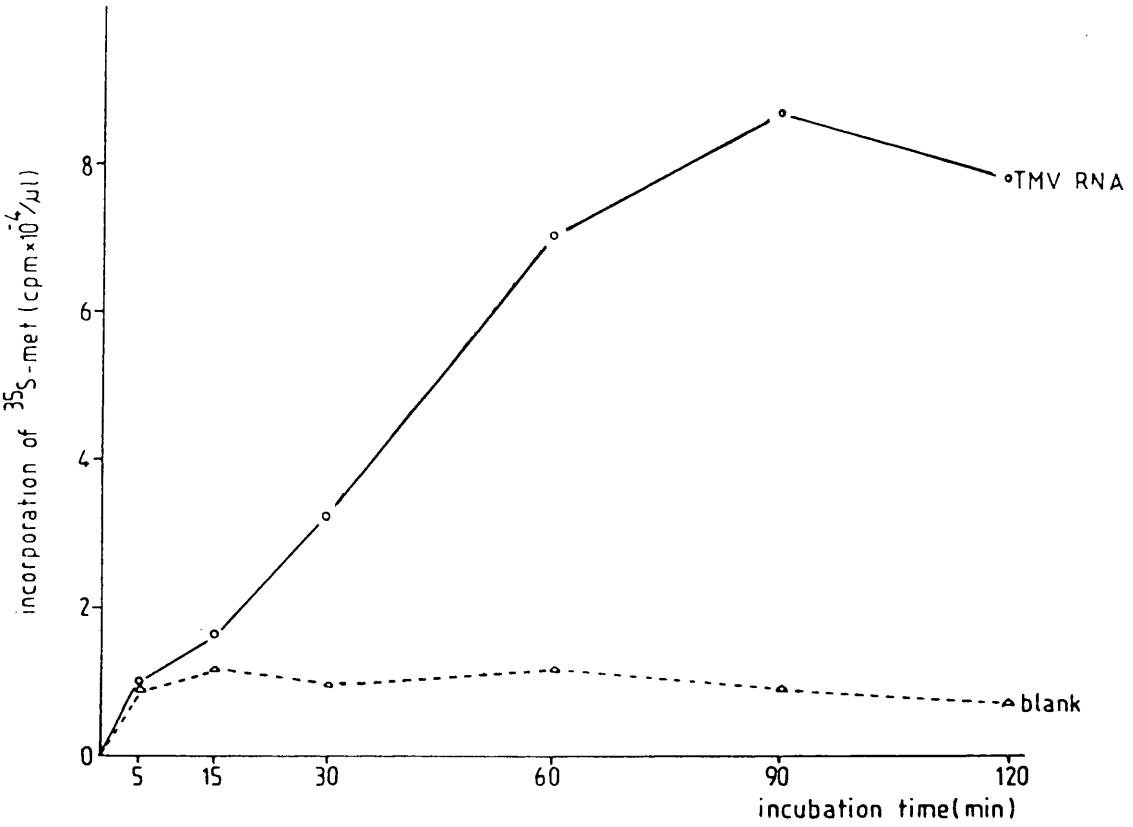


Fig. 4.3. Time-course of protein synthesis directed by TMV RNA in wheat germ lysate cell-free system.

Table 4.1. Incorporation of radioactive amino acid directed by TMV RNA.

Radioactive amino acid	Radioactivity incorporation control(cpm)	TMV RNA(cpm)	Incorporation ratio (TMV RNA/control)
^{35}S-Met	6,038	157,199	26.04
^{3}H-Phe	400	10,878	27.19
^{14}C-Leu	220	5,306	24.12

11 µl reaction mixture were incubated for 60 min, at 30° C with ^{35}S-Met (136µCi/ml), ^{3}H-Phe (180µCi/ml), and ^{14}C-Leu (50µCi/ml) and contained 0.1 µl of TMV RNA. The percentage of reticulocyte lysate in reaction mixture was 69%. Control, reactions were incubated in the absence of TMV RNA. Duplicate 1 µl samples of reaction mixture were counted for radioactivity incorporation after TCA precipitation.

short polypeptide form.

In our experiments, wheat germ lysate in the presence of 80μM spermidine phosphate and 3.05mM Mg(OAc) actively translated TMV RNA but its efficiency to synthesize high M.W. protein(176K) was rather lower than rabbit reticulocyte lysate.

4.1.2. Protein Synthesis in Rabbit Reticulocyte Lysate Cell-free Systems.

The optimum conditions and the procedure were as described in section 2.2.6.

4.1.2.a. Translation of TMV RNA.

When added to reticulocyte lysate (80%,V/V), TMV RNA strongly stimulated the incorporation of ^{35}S-Met into protein. Time course studies of the reaction at 30°C indicated a lag period of 5 min, followed by greatly enhanced incorporation of radioactivity for 30 min which then levelled off until 90 min of incubation (Fig.4.4.a). The incorporation of ^{35}S-Met was dependent on the amount of added TMV RNA and was saturated at about ½ the relative amount of TMV RNA(or 25μl/ml) (Fig.4.4.b.). Under optimal conditions, incorporation of ^{35}S-Met directed by TMV RNA was more than 100 fold that of the control incubation at the same time, in the absence of exogenous mRNA. In addition, this reticulocyte lysate cell-free system was very active in translation of TMV RNA and showed a significant difference in incorporation of radioactivity at a concentration of TMV RNA as low as 1μl/ml.

With different radioactive amino acids (^{35}S-Met, ^{14}C-Leu and ^{3}H-Phe), the stimulation of incorporation directed by TMV RNA at a concentration of 9.1μl/ml) was similar and about 24-27 fold higher than the control. The ratio of these radioactivity incorporationsis shown in Table 4.1.

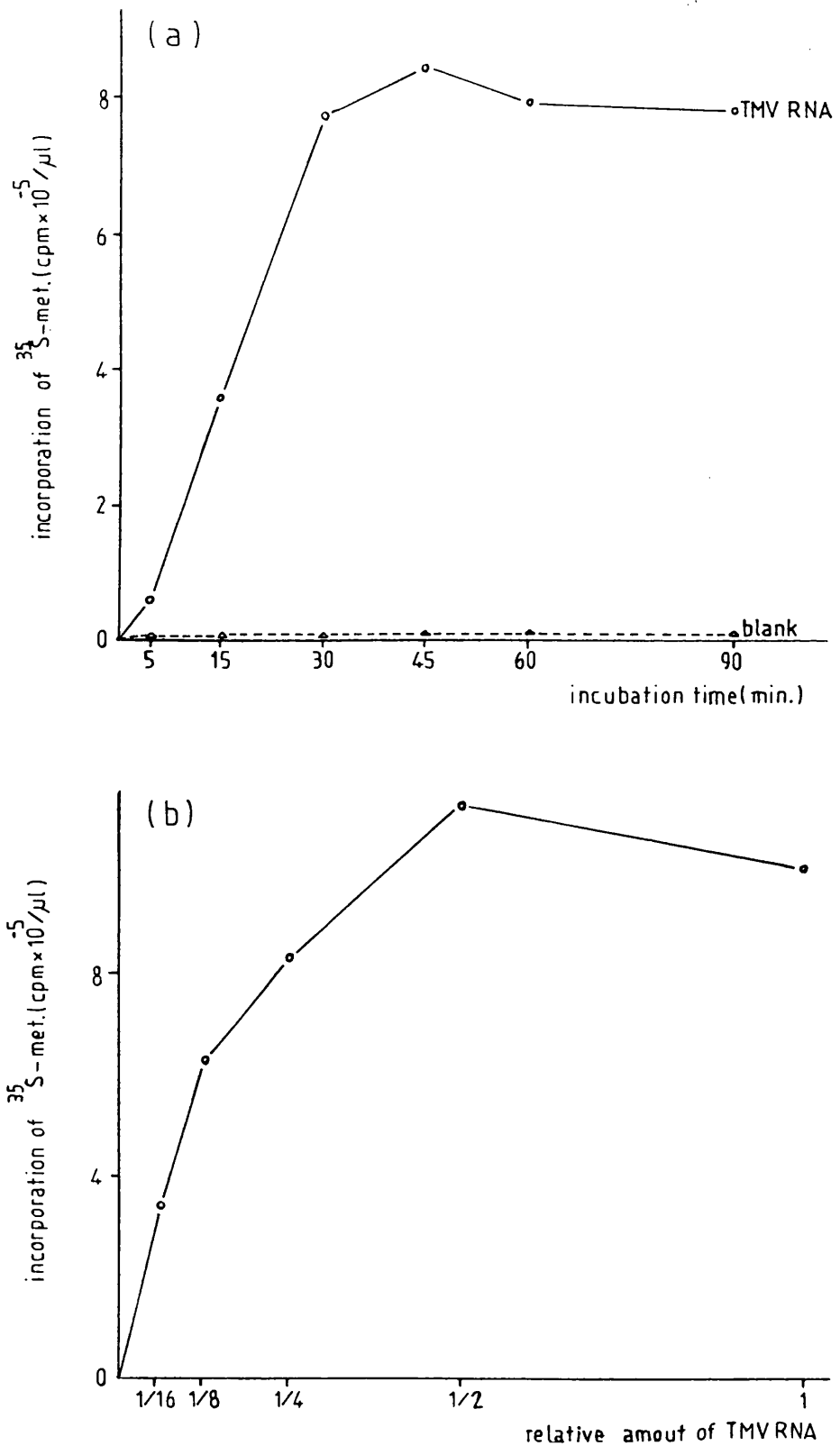


Fig. 4.4. Translation of protein directed by TMV RNA in rabbit reticulocyte lysate. The incubations were carried out at 30°C with ^{35}S-Met.

a) Time-course experiment, using 1.5μl of TMV RNA in 25μl of reaction mixture.

b) Effect of TMV RNA concentration on protein synthesis activity (60 min incubation).

Fig. 4.5.

a) SDS-PAGE of TMV protein synthesized in rabbit reticulocyte lysate. The radioactivity content in each 1.5mm gel slice was determined after solubilizing the gel in hyamine hydroxide and soluene 350. The M.W. of protein was estimated from calibration curve, using Rf value. Control, without mRNA (▵——▵) and added TMV RNA (•——○).

b) Fluorogram of translation product directed by TMV RNA and globin mRNA in wheat germ lysate and rabbit reticulocyte lysate cell-free system and separated on 5-15% SDS-PAGE.

lane 1: without exogenous mRNA.

" 2: added TMV RNA and incubated with ^{35}S-Met in rabbit reticulocyte lysate cell-free system.

" 3: added TMV RNA and incubated with ^{14}C-Leu in rabbit reticulocyte lysate cell-free system.

" 4: added TMV RNA and incubated with ^{3}H-Phe in rabbit reticulocyte lysate cell-free system.

" 5: added TMV RNA in wheat germ lysate and incubated with ^{35}S-Met.

" 6: added TMV RNA in wheat germ lysate and incubated with ^{35}S-Met.

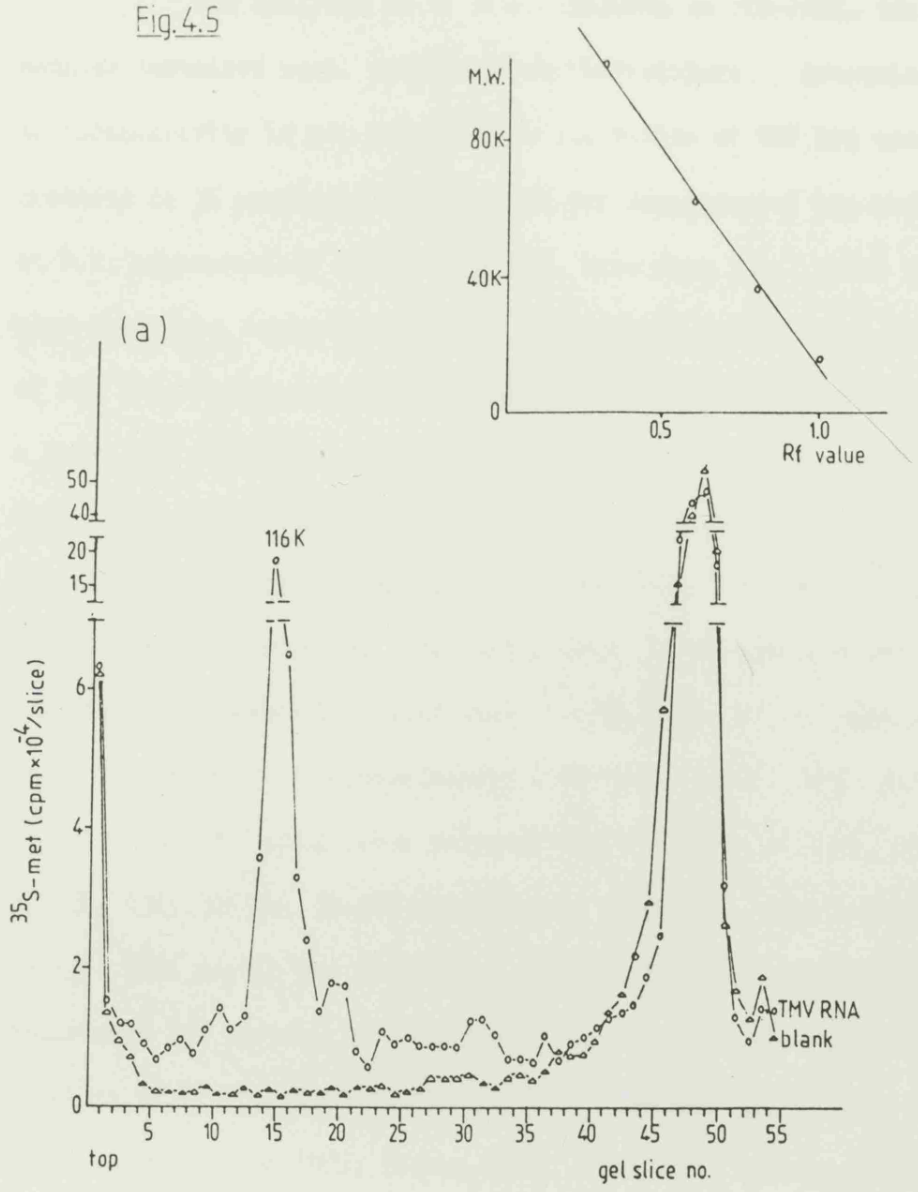
" 7: added globin mRNA in reticulocyte lysate and incubated with ^{35}S-Met.

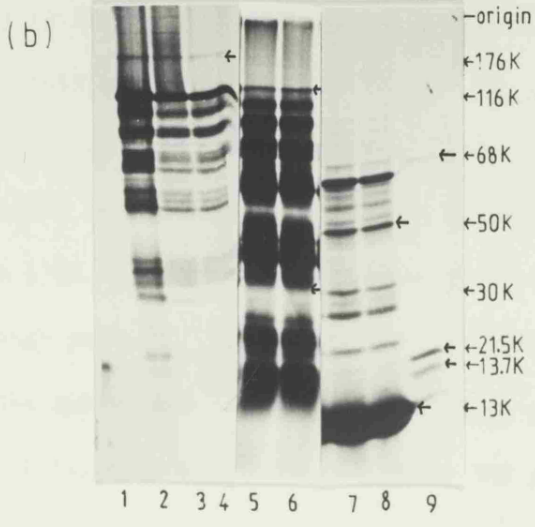
" 8: added globin mRNA in reticulocyte lysate and incubated with ^{35}S-Met.

" 9: standard ^{14}C-BSA, ^{14}C-trypsin inhibitor, and ^{14}C-RNase.

Exposure: 40,000 to 200,000 cpm; 4,6,and 10 days; -70° C.

Fig. 4.5


M.W.
80K
40K
0
0.5
1.0
Rf value
(a)
116K
^{35}S-met (cpm×10^{-4}/slice)
TMV RNA
blank
top
gel slice no.


(b)
origin
176K
116K
68K
50K
30K
21.5K
13.7K
13K
1 2 3 4 5 6 7 8 9

For analysis of in vitro products on SDS-PAGE, the gel samples contained equal volumes of reaction mixture. Determination of radioactivity in gel slices after separation of TMV RNA translation products on 5% acrylamide cylindrical gel demonstrated the major peaks at M.W. approximately 116K and at M.W. less than 10K, which was also shown in a control(without TMV RNA) (Fig.4.5.a.). The fluorogram of TMV RNA products separated on 5-15% SDS-PAGE, slab gel indicated a large and complex distribution of polypeptides of M.W. ranging from 21K to 176K (Fig.4.5.b. lane 2-4). The profile of TMV proteins labelled with different radioactive amino acids (^{35}S-Met, ^{14}C-Leu and ^{3}H-Phe) was similar except that TMV protein bands appeared with increased intensity in samples incubated with ^{35}S-Met (Fig. 4.5.b. lane 2). A major product of M.W. approximately 116K and a readthrough product of M.W. 176K including other polypeptides with M.W. of 100K, 95K, 70-75K, 65K, 50-55K, 30-38K and 20K were observed (Fig. 4.5.b. lane 2-4). This result was similar to other investigators who showed that translated TMV protein in reticulocyte lysate included two large polypeptides of M.W. approximately 110K and a readthrough product of 160K (Pelham and Jackson,1976; Pelham,1978). A large reproducible number of discrete polypeptides of M.W. below 110K have frequently been observed at varying intensities during translation of TMV RNA in rabbit reticulocyte lysate cell-free systems(Pelham and Jackson,1976; Beier et al.,1980; Kurkinen,1981; Goelet and Kahn,1982). The M.W. of two large polypeptides varied between different investigations and was referred to as 110K, 130K and 140K formajor product, and 160K and 165K for a readthrough product. A precise M.W. of 126K and 183K was deduced from the nucleotide sequences (Goelet et al.,1982). From our resultsthe average valuesof M.W. of these TMV proteins differed slightly from their precise values and were 116 $\pm$ 5.99 and 176 $\pm$ 6.49,

respectively for a major protein and their readthrough product.

A smaller product of 30K-38K was also shown in Fig.4.5.b. lane 2-4. These proteins might be a set of proteins with M.W. of 30K, 29K, and 28K(Beachy and Zaitlin,1977; Hunter et al.,1983). The synthesis of 30K protein was found to be different in amount and differed slightly in size for each virus RNA, TMV vulgare or U_1,TMV dahlemense, TMV U_2 and cowpeastrain of TMV (Beier et al.,1980).

The coat protein of M.W. 17.5K was not detected in the translation product of TMV RNA in reticulocyte lysate (Fig. 4.5.b. lane 2-4). Hunter et al. (1976) and Beier et al. (1980) have shown that coat protein in virus RNA was not a major product and was never detected in reticulocyte lysate and xenopus laevis oocyte systems except using cowpea strain of TMV RNA that showed a protein with the same electrophoretic mobility as coat protein.

4.1.2.b. Translation of globin mRNA.

In 40% (V/V) of reticulocyte lysate, rabbit globin mRNA stimulated the incorporation of ^{35}S-Met into TCA-precipitable product approximately 10 fold over the control, without exogenous mRNA. In a time-course, incorporation directed by globin mRNA appeared to increase linearly for about 60 min until terminated at 90 min (Fig. 4.6.a). Increasing the mRNA concentration resulted in a greater amount of ^{35}S-Met incorporation and was saturated at about 0.075µg of globin mRNA in 10µl of reaction mixture. (Fig.4.6.b.)

5-15% SDS-PAGE analysis of the translation product of globin mRNA with a fluorography technique revealed the presence of a major product at a M.W. of approximately 13K which had about the same electrophoretic mobility as globin protein (Fig.4.5b. lane 7 and 8). By this sensitive method, several minor components at M.W. of 50-65K,

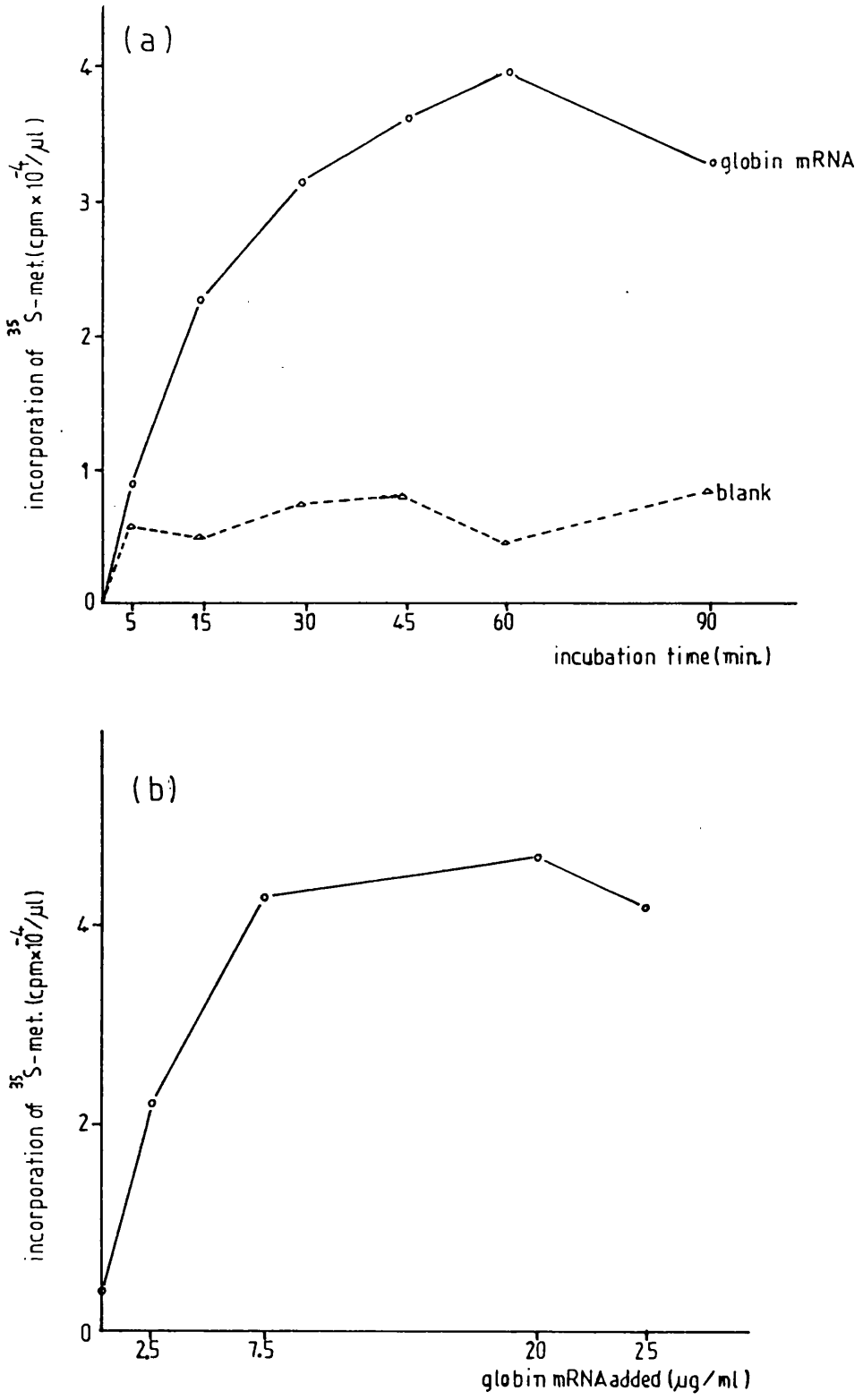


Fig. 4.6. Translation of globin mRNA in rabbit reticulocyte lysate. The reaction mixture was incubated at 30° C and radioactivity was determined on 1µl reaction mixture.

a) Time-course experiment using 0.5µg of globin mRNA in 25µl reaction mixture.

b) Response of incorporation to added globin mRNA concentration (incubation time 60 min).

35 -40K and 23K were also observed in the translation products of globin mRNA. This result might suggest that globin mRNA was not cleaved since a high amount of globin protein was synthesised in the cell-free system. However, globin mRNA might not be in a pure form and may result in the synthesis of many sizes of polypeptides.

4.1.2.c. Translation of poly(A)$^+$RNA from rat liver, K562 and NS 1 cells

Total RNAs were extracted from rat liver, K562 and NS 1 cells using a phenol-chloroform deproteinization procedure and yielded poly(A)$^+$RNA after affinity chromatography on oligo(dT) cellulose column. The results of isolation and purification of these poly(A)$^+$RNAs were previously presented in chapter 3, section 3.2.1.e.

The ability of these poly(A)$^+$RNAs to direct protein synthesis in the rabbit reticulocyte lysate cell-free system was measured using the incorporation of radioactive amino acid into acid-precipitable protein.

In rabbit reticulocyte lysate (80%,V/V), the incorporation of ^{35}S-Met in response to rat liver poly(A)$^+$RNA was dependent on added poly(A)$^+$RNA and incubation time (Fig.4.7.a. and 4.7.b., respectively). Under the conditions used, it was not possible to add saturating amounts of RNA. Specific incorporation, approximately 4 fold higher than the control without exogenous RNA was achieved with 5µg of poly(A)$^+$RNA in 10µl reaction mixture (Fig.4.7.a.).

In 68% (V/V) reticulocyte lysate, the K562 poly(A)$^+$RNA and NS 1 poly(A)$^+$RNA at the concentration of 0.1 A_{260} units/11µl reaction mixture stimulated incorporation of ^{35}S-Met approximately 8 fold and 4 fold, respectively, over the endogenous reticulocyte lysate protein synthesis without added RNA. The incorporation of ^{35}S-Met directed by either K562 poly(A)$^+$RNA or NS 1 poly(A)$^+$RNA was

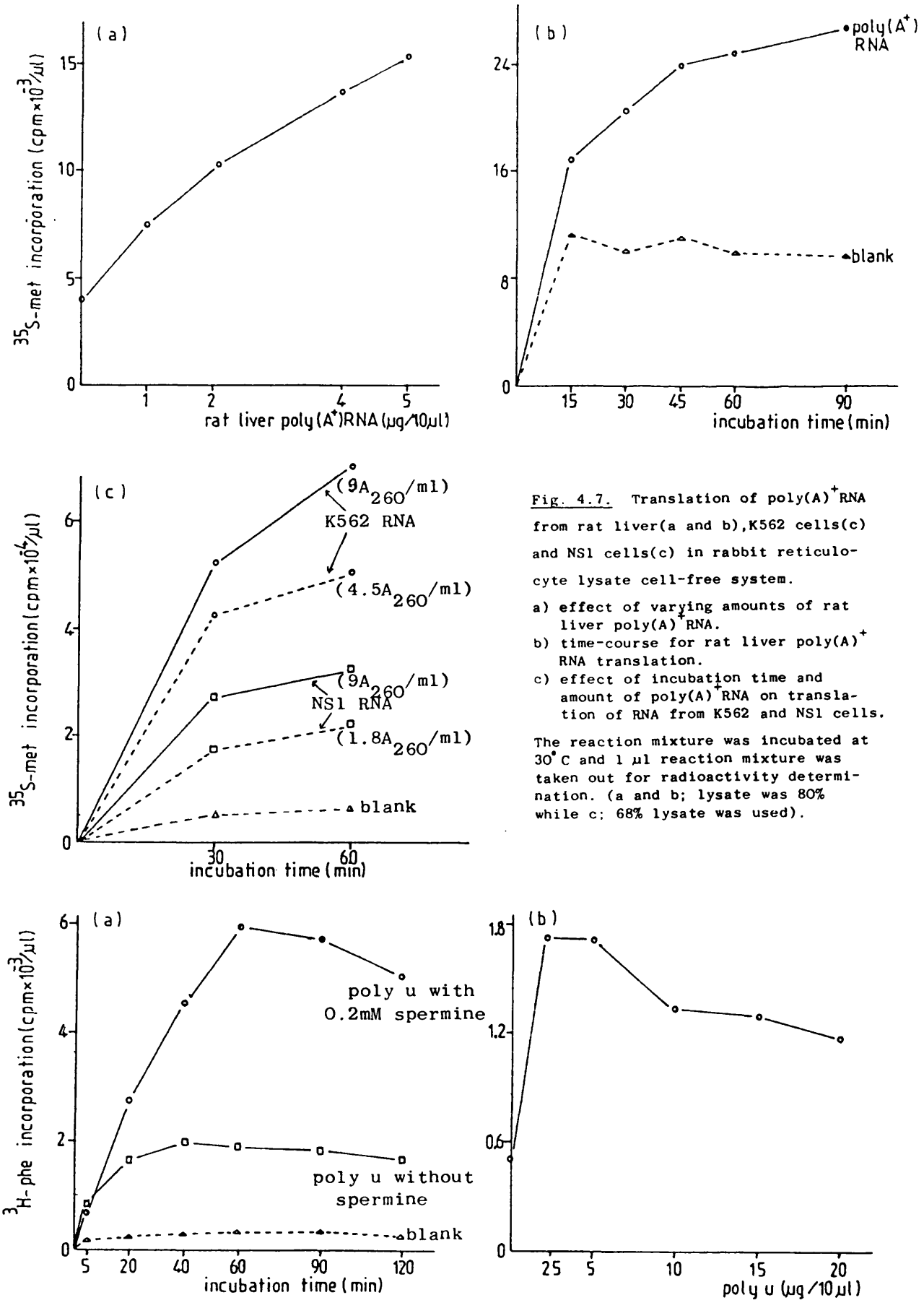


Fig. 4.7. Translation of poly(A)$^+$RNA from rat liver(a and b),K562 cells(c) and NS1 cells(c) in rabbit reticulocyte lysate cell-free system.

a) effect of varying amounts of rat liver poly(A)$^+$RNA.
b) time-course for rat liver poly(A)$^+$ RNA translation.
c) effect of incubation time and amount of poly(A)$^+$RNA on translation of RNA from K562 and NS1 cells.

The reaction mixture was incubated at 30° C and 1 µl reaction mixture was taken out for radioactivity determination. (a and b; lysate was 80% while c; 68% lysate was used).

Fig. 4.8. Effect of incubation time (a) and concentration of poly u (b) on polyphe synthesis in rabbit reticulocyte lysate. All incubations were carried out at 30° C with ^{3}H-Phe and 1 or 2 µl of reaction mixture was used to determined radioactivity. In (b), 66% lysate in the absence of spermine HCl were used and incubated for 90 min. Lysate concentration was 86% in (a) experiment.

also proportional to incubation time, and concentration of added poly(A)$^+$ RNA as shown in Fig 4.7.c.

4.1.2.d. Translation of poly u.

In rabbit reticulocyte lysate (86%,V/V), the incorporation of ^{3}H-Phe directed by poly u was approximately 7 fold over the control without poly u and the efficiency of poly u translation was increased to approximately 20 fold when adding 0.2mM spermine hydrochloride into the reaction mixture (Fig.4.8.a.). Incorporation of ^{3}H-Phe into polypeptide increased linearly with time up to 60 min and then leveled off (Fig 4.8.a.). The incorporation of radioactivity into polypeptide increased with increasing amount of poly u and maximum incorporation occurred between 0.23 and 0.46 mg/ml of added poly u; further increase in poly u caused a decrease in incorporation (Fig.4.8.b.).

4.2. Conclusion.

Many cell-free protein synthesizing systems,such as reticulocyte lysate, wheat germ lysate, Krebs II ascite cells, mouse L cells and xenopus leavis oocytes have shown to be able to translate mRNAs from several different sources. Some of these systems were limited by the efficiency of translation, the degree of fidelity of translation and high levels of endogenous protein synthesis.

Wheat germ lysate and rabbit reticulocyte lysate cell-free systems were chosen to study,for their efficiencies in translation of several mRNAs. The results presented here demonstrate that these two cell-free systems are capable of translating poly u, TMV RNA, globin mRNA and poly(A)$^+$RNA from rat liver, K562 and NS 1 cells. Although these mRNAs and poly u were able to direct the synthesis of

their various protein products in cell-free systems, the relative efficiencies of translation for each messenger were apparently different and required their individual optimization.

The wheat germ protein synthesizing system has distinct advantages over other cell-free systems. These advantages involved the ease and rapid preparation in large quantities, low cost, high translational efficiency with ability to synthesize large polypeptides (M.W. >50K or as high as 140K) and, in particular, a low level of endogenous mRNA activity (Roberts and Paterson,1973; Roberts et al., 1974; Marcu and Dudock,1974 and Benveniste et al.,1976). In our work, poly u and TMV RNA were translated in a wheat germ lysate cell-free system and emphasis was placed on finding a maximum rate of translation. The results showed that this cell-free system was very active in synthesis of polyphe and TMV protein in response to poly u and TMV RNA, respectively. The variation in the optimum conditions was found to relate to the type of added RNA.

In translation of poly u, the maximum incorporation of ^{3}H-Phe into polyphe was achieved by using 7.6-10.8 mg/ml of wheat germ lysate (protein concentration), 64mM KCl, 2.5mM Mg(OAc), 0.2mM spermidine hydrochloride, 80μCi/ml of ^{3}H-Phe and 0.8-1.05 mg/ml of poly u whereas the concentration of other components was previously shown in method section 2.2.6. This optimization for poly u translation in wheat germ lysate was slightly different from previous works by Abraham et al. (1979), Algranati (1980), Igarashi et al. (1979) and Igarashi et al. (1982). Poly u directed radioactivity incorporation was 8-10 fold greater than that of controls,without poly u. When adding an optimal concentration of spermidine hydrochloride (0.2mM) into the reaction mixture of wheat germ lysate, the optimum Mg(OAc) concentration was reduced to 2.5mM and total radioactivity incorporation

was increased by about 3 fold from using only optimal concentration of Mg(OAc). This result confirmed the previous studies which found that highly activated poly u translation including the fidelity of translation in wheat germ lysate cell-free system was achieved when Mg^{2+} concentration was partially substituted by polyamine and this stimulatory effect could not be equalled by any amount of Mg^{2+} (Hunter et al.,1977; Igarashi et al.,1979; Abraham et al.,1979; Watanabe et al.,1981). Poly u directed the synthesis of high M.W. polyphe as shown by a large distribution of polypeptides with M.W. higher than 45K to 90K after analysis on 7.5% SDS-PAGE. Most of the incorporated radioactivity was found as a single high M.W. peak when the translation product of poly u in wheat germ lysate was analysed by sucrose gradient sedimentation. This result indicated that wheat germ lysate had a high efficiency to synthesize large polypeptides in response to poly u. This optimum condition of wheat germ lysate was used to check the translational activity of isolated poly$(A)^+$RNA from tissue or cells as described in chapter 3.

The wheat germ lysate reaction mixture (in the presence of 80µM spermidine hydrochloride and 3.07mM Mg(OAc), 50µM each of 19 amino acid except met and 26 A_{260} units/ml of nuclease-treated wheat germ lysate) was used to translate TMV RNA and gave an increase in radioactivity incorporation of approximately 9 fold over the background at 5.3µl/ml of TMV RNA. By analysis on 5-15% SDS-PAGE, the distribution of protein with M.W. between 10K and 120K (or precised M.W. of 126K) was seen. High amounts of low M.W. products and no synthesis of protein at M.W. of 160K (or precised M.W. of 183K) were found when the protein profile was compared with translation products of TMV RNA in rabbit reticulocyte lysate cell-free system. Support for this result was obtained from a previous study in which the highest M.W

of TMV protein synthesized in the presence of 1.5mM Mg^{2+} and 0.66mM spermine was 110K (Abraham et al.,1979). Roberts and Paterson(1973) and Hunter et al.(1977) also demonstrated that a large number of discrete polypeptides ranging in M.W. from 10K up to a maximum of 140K of TMV product in wheat germ lysate which was a major product of TMV RNA in other systems (Pelham and Jackson,1976; Knowland,1974). Marcu and Dudock (1974) demonstrated a maximum M.W. of 80K from the analysis of TMV protein from wheat germ lysate protein synthesizing system on 14% PAGE. The accumulation of incomplete chains during translation of TMV messenger in wheat germ lysate was suggested to be due to premature termination (Abraham et al.,1979), decreased elongation (Hunter et al.,1977), a reduction in the initiation step at the level of binding of aminoacyl tRNA to ribosomes(Igarashi et al.,1982) and the presence of RNase itself (Hunter et al.,1977; Scheele and Blackburn,1979). The higher yield of full length product of protein synthesis in wheat germ lysate suggests that fidelity of translation might be achieved by partial substitution of Mg^{2+} concentration with polyamine (Hunter et al.,1977 ; Abraham et al.,1979; Igarashi et al.,1982), increasing K^{+} concentration to higher than that for optimal protein synthesis (Benveniste et al.,1976; Schmeckpeper et al.,1974) and by adding RNase inhibitor to the reaction mixture (Hiranyavasit and Kusamran,1983).

Rabbit reticulocyte lysate is a mRNA-dependent protein synthesizing system after removal of endogenous translatable mRNA by preincubation of the lysate with micrococcal nuclease in the presence of Ca^{2+}, as described by Pelham and Jackson (1976). This lysate had an extremely low rate of endogenous protein synthesis and appeared to be highly selective for translation of various mRNAs (Pelham and Jackson,1976; Chu and Rhoads,1980). Optimum concentrations of exogenous

mRNAs (TMV RNA, globin mRNA, poly(A) RNA from rat liver, K562 and NS 1 cells, and poly u) and an optimum incubation time for maximum protein synthesis in rabbit reticulocyte lysate was determined. The percentage of lysate(V/V)was varied between 40% and 86% dependent upon the concentrations of the messengers used. The results showed that there was a difference in the efficiency of translation for each of these messengers including their optimal concentration of added mRNA in rabbit reticulocyte lysate cell-free system. Endogenous protein synthesis in controls was less than 5% maximum of radioactivity incorporation. The rate of incorporation of radioactivity into protein was approximately 100fold, 20 fold, 10 fold, 8 fold and 4 fold above the controls when TMV RNA (50µl/ml), poly u (0.5mg/ml), globin mRNA (7.5µg/ml), K562 poly(A)$^+$RNA (9 A_{260}units/ml) or poly(A)$^+$RNA from rat liver(500µg/ml) and NS 1 cells (9 A_{260} units/ml) were added,respectively into the reaction mixture. These messengers have been translated effectively in this cell-free system although none stimulated protein synthesis as effectively as TMV RNA.

Translation of TMV RNA in rabbit reticulocyte lysate was efficient and equivalent to the data presented by Pelham and Jackson (1976), Pelham (1978), Beier et al. (1980) and Kurkinen (1981). A large number of polypeptides ranging in M.W. from 21K to 176K were indicated from analysis of in vitro synthesized TMV protein on 5-15% SDS-PAGE (Fig.4.5.b. lane 2-4) This rabbit reticulocyte lysate also appeared to be highly selective for globin mRNA translation. The efficiency for translation of globin mRNA was at least similar to that reported by Pelham and Jackson (1976) and Ravel and Groner (1978) (in reticulocyte lysate) and Roberts and Paterson (1973) and Schmeckpeper et al. (1974) (in wheat germ lysate). The in vitro product on SDS-PAGE (Fig. 4.5.b. lane 7 and 8) indicated a major band which had

a mobility the same as globin protein (M.W. of about 13K) and also showed by Robertsand Paterson (1973),Schmeckpeper et al.(1974) and Pelham and Jackson (1976). This rabbit reticulocyte lysate appeared to stimulate the synthesis of protein in response to poly(A)$^+$RNA from rat liver, K562 and NS 1 cells and poly u. Different mRNA species translated with varying efficiency,and TMV RNA was the most active mRNA in directing incorporation of ^{35}S-Met in rabbit reticulocyte lysate cell-free systems. Therefore, to determine the control of the synthesis of protein at translation level by ANAs in a cell-free system TMV RNA and rabbit reticulocyte lysate were the main systems. Other RNAs such as globin mRNA, poly u and K562 and NS 1 poly(A)$^+$RNA were used in alternative systems including the wheat germ lysate cell-free system.

Chapter Five Effect of ANAs on RNA translation in rabbit reticulocyte lysate and wheat germ lysate cell-free systems.

5.1. Methods

5.1.1. Effect of ANAs on protein synthesis in cell-free translation systems.

-The rabbit reticulocyte lysate cell-free system.

-The wheat germ lysate cell-free system.

5.1.2. Effect of PBS concentration on translation of TMV RNA or globin mRNA in rabbit reticulocyte lysate and wheat germ lysate cell-free systems.

-Conductivity measurement.

-Effect of PBS concentration and ANA on protein synthesis.

5.1.3. Comparison of the effects of ANA and inhibitors on protein synthesis.

5.1.4. Preparation of specific antiRo and antiLa by affinity chromatography.

-Coupling of antigen Ro or La to Sepharose 4B

-Purification of specific antiRo or antiLa by using antigen Ro- or antigen La-Sepharose 4B column.

5.1.5. Determination of specific antigenic proteins of ANAs by immunoblotting technique.

5.1.5.a. Electrophoretic transfer of proteins from PAGs to nitrocellulose sheets.

5.1.5.b. Detection of protein on nitrocellulose by staining with amido black.

5.1.5.c. Immunological detection of antigen on nitrocellulose using peroxidase-conjugated anti-humanIgG.

-Pretreatment and storage of nitrocellulose sheet containing immobilized polypeptides.

-Detection of antigen-antibody complex using peroxidase-conjugated anti-humanIgG.

5.2. Results

5.2.1. Specificity and concentration of IgG solution from normal individuals and SLE patients.

5.2.2. Effect of ANAs on TMV RNA translation in rabbit reticulocyte lysate cell-free system.

5.2.2.a. Effect of concentration of ANA and incubation time on TMV RNA translation.

5.2.2.b. Analysis of translation product on SDS-PAGE.

5.2.2.c. The inhibitory effect of ANAs on translation of TMV RNA.

5.2.2.d. Effect of salt concentration on TMV RNA translation.

5.2.2.e. Comparison of inhibition of TMV RNA translation by ANAs and other protein synthesis inhibitors.

5.2.2.f. Effect of specific antiRo- and antiLa-antibodies on TMV RNA translation.

5.2.3. Effect of ANAs on translation of various RNA templates in rabbit reticulocyte lysate cell-free system.

5.2.3.a. The effect of ANAs on translation of globin mRNA.

5.2.3.b. The effect of ANAs on translation of poly(A)$^+$RNA from K562 and NS1 cells.

5.2.3.c. Translation of poly u affected by ANAs.

5.2.4. Effect of PBS concentration on translation of globin mRNA in rabbit reticulocyte lysate and inhibitory mechanism of ANA.

5.2.4.a. Effect of PBS concentration on globin mRNA translation.

5.2.4.b. Inhibition of globin protein synthesis by ANA.

5.2.5. Effect of ANAs on TMV RNA translation in wheat germ lysate.

5.2.5.a. Inhibitory effect of ANAs on TMV RNA translation.

5.2.5.b. Effect of PBS concentration on TMV RNA translation.

5.2.6. Detection of the antigenically active protein in cell lysates using immunoblotting technique.

5 Effect of ANAs on RNA translation in rabbit reticulocyte lysate and wheat germ lysate cell-free systems.

The results of effects of ANAs on protein synthesis will be discussed in the next two chapters. In chapter 5, we have used the cell-free systems (rabbit reticulocyte lysate and wheat germ lysate) with various RNA templates and studied the effect of ANAs on translation. The effect of ANAs was further determined on protein synthesis in intact cells as described in chapter 6.

5.1. Methods.

5.1.1. Effect of ANAs on protein synthesis in cell-free translation systems.

The optimal conditions used to synthesize proteins in the cell-free systems were described in section 2.2.6. including determination of radioactivity. Preparation and characterization of IgG fractions from normal individuals and SLE patients were described in sections 2.2.3. and 2.2.5., respectively. SDS-PAGE and fluorography were also described in section 2.2.7.

The rabbit reticulocyte lysate cell-free system.

The control was a sample in which PBS or normal IgG solution at a concentration of 0.2µg/µl reaction mixture) was added to the reaction mixture. In experimental tubes, each ANA sample (at 0.55 to 2.2µg IgG/tube) was incubated with 11µl reaction mixture at 30°C. The percentage of lysate (V/V) was 65-68% for TMV RNA, 60% for globin mRNA and NS 1 poly(A)$^+$RNA, 68% for K562 poly(A)$^+$RNA and 66% for poly u. Duplicate 1µl samples of reaction mixture were withdrawn to determine acid-insoluble radioactivity at 30 and 60 min of incubation and the rest of the reaction mixture was used for gel analysis.

The wheat germ lysate cell-free system.

The reaction mixture contained the components described in section 2.2.6, except that IgG (at 0.2µg/µl reaction mixture) from a normal individual, or SLE patient was added to control or experimental tubes, respectively. The reaction mixture was incubated at 25°C for 30, 60 and 90 min. At each of these incubation times, duplicate 1µl samples of reaction mixture were precipitated with TCA and radioactivity was counted. The rest of the reaction mixture was further analyzed on SDS-PAGE.

5.1.2. Effect of PBS concentration on translation of TMV RNA or globin mRNA in rabbit reticulocyte lysate and wheat germ lysate cell-free systems.

Conductivity measurement.

10µl of PBS solution at various concentrations or diluted IgG solution from normal individuals and SLE patients were added into 20ml of double distilled water, mixed well and the conductivity was measured using a Radiometer type CDM 104 conductivity cell.

Effect of PBS concentration and ANA on protein synthesis.

In this experiment IgG solutions from both normal individuals and SLE patients were diluted with PBS instead of distilled water. IgG samples or PBS at various concentrations were added into the reaction mixture and incubated at 25°C for wheat germ lysate, and 30°C for reticulocyte lysate. At 30 and 60 min of incubation, duplicate 1µl samples of reaction mixture was withdrawn for radioactivity determination and the rest of the mixture was kept for gel analysis.

5.1.3. Comparison of the effects of ANA and inhibitors on protein synthesis.

Aurin tricarboxylic acid and cycloheximide inhibited protein synthesis at the initiation and elongation steps, respectively

(Lodish et al.,1971; Mathews, 1971;Bathurst et al.,1980). These protein synthesis inhibitors were used to compare their effect on protein synthesis in reticulocyte lysate with ANA in a time-course experiment. The reaction mixture (which contained 81.5% lysate) was incubated at 30°C and duplicate 1µl samples of reaction mixture were taken out for radioactivity determination at 5 and 10 min. At 10 min incubation, 0.2µg/µl IgG solution, 50µM cycloheximide and 50µM aurin tricarboxylic acid (a final concentration) were added which resulted in a final lysate concentration of 76.5%. The assay was continued and duplicate 1µl samples of reaction mixture were taken to determine radioactivity at 15, 20, 30 and 60 min of incubation. The rest of reaction mixture was further analyzed on SDS-PAGE.

5.1.4. Preparation of specific antiRo and antiLa by affinity chromatography.

Coupling of antigen Ro or La to Sepharose 4B.

Antigen Ro and antigen La were provided by Dr. P. Skinner, Royal National Hospital for Rheumatic Diseases, Bath. Both antigens were prepared by using their antibody affinity chromatography with antiRo (Frayne)- and antiLa (Buffalo)-Sepharose 4B columns. Activation of Sepharose 4B with cyanogen bromide and coupling of antigen were performed according to the method described by March et al. (1974).

Sepharose 4B (about 4 grams wet weight) was washed on a sintered glass funnel with 50ml of H_2O, suspended in 10ml of 2M sodium carbonate and mixed slowly. The mixing was changed to a fast rate before adding 0.5ml of cyanogen bromide solution (1.150g/ml in acetonitrile) and stirring was continued for 1-2 min. The mixture was then poured on to a coarse sintered glass funnel and washed with 50ml of H_2O and 50ml of PBS.

About 1.5g of dry activated Sepharose 4B were added to

antigen Ro in PBS(1.6mg in 7ml PBS)or antigen La in PBS (1.7mg in 6ml PBS). The reactants were mixed slowly in a rotary mixer at 4°C for 22 hr, and filtered on a sintered glass funnel. This Sepharose 4B was then resuspended in 0.2M sodium carbonate pH8,containing 1M glycine to block any unreacted sites and stirred slowly at 4°C overnight. The mixture was again filtered on a sintered glass funnel and washed with PBS. This Sepharose 4B-coupled with antigen Ro or La was packed into a column (length 5cm, diam. 1cm), washed with 3M guanidine-HCl pH7 and equilibrated in PBS. The coupling of antigen Ro and antigen La to Sepharose 4B was 30.4% or (0.49mg/ml of Sepharose 4B) and 16.5% (or 0.28mg/ml of Sepharose 4B), respectively.

Purification of specific antiRo or antiLa by using antigen Ro- or antigen La-Sepharose 4B column.

The IgG-antiRo solution or IgG-antiLa solution was applied to the antigen-Sepharose-4B column and the unbound material was eluted with PBS. The bound material was eluted with 3M guanidine-HCl pH7 and immediately dialysed against PBS, at 4°C, overnight.

This specific antiRo antibody fraction or antiLa antibody fraction was tested for specificity by an ELISA method and used to to test for effects on protein synthesis in cell-free systems.

5.1.5. Determination of specific antigenic proteins of ANAs by immunoblotting technique.

5.1.5.a. Electrophoretic transfer of Proteins from PAGs to nitrocellulose sheets.

The method employed was adapted from the procedure of Towbin et al.(1979). Nitrocellulose sheets(0.2µM pore size, Schleicher and Schüll BA83) were briefly wetted with 30mM phosphate buffer pH6.5. After running electrophoresis of antigen (both crude and purified form) and wheat germ lysate or reticulocyte lysate on

5-15% SDS-PAG, the gel was briefly washed in 30mM phosphate buffer pH6.5 and placed on a scouring pad (Scotch-brite) which was supported by a stiff plastic grid. The wet nitrocellulose was put on the gel and all air bubbles carefully removed. A second pad and plastic grid were added and held tightly before putting into an electrophoresis chamber (a Trans-blot cell) with the nitrocellulose sheet towards the anode. The chamber contained about 3 litres of 30mM phosphate buffer pH6.5. Electrophoresis was run at 15 volts for 20-22 hr at room temperature.

5.1.5.b. Detection of protein on nitrocellulose by staining with amido black.

After blotting, the nitrocellulose sheet was stained in 0.1% amido black (in 50% methanol containing 10% acetic acid) for 10 to 15 min and destained in 50% methanol containing 10% acetic acid until a clear background was obtained. This method was adapted from the procedure of Schaffner and Weissmann (1973).

5.1.5. c. Immunological detection of antigen on nitrocellulose using peroxidase-conjugated anti-human IgG.

Pretreatment and storage of nitrocellulose sheet containing immobilized polypeptides.

Gelatin, BSA, tween 20 and casein, (all in 50mM Tris-HCl pH7.4 containing 0.9% NaCl)were tested for their efficiences in preventing the non-specific binding of antibody and other proteins to the nitrocellulose paper. Casein, BSA, and tween 20 gave a clear background, and the sensitivity in detecting the immune complex on casein and BSA treated nitrocellulose sheets was higher than tween-20 treated sheets. Using casein was an economical method, therefore, 1% casein (in 50mM Tris-HCl pH7.4 containing 0.9% NaCl or in PBS) was used in all the incubation and washing steps to prevent non-specific binding.

The nitrocellulose sheet was treated in 1% casein in PBS for at least 4 hr at 37°C or overnight at room temperature before processing for immunological detection or storage.

For storage, the sheet was briefly rinsed in PBS and blotted dry between 2 sheets of 3 mm filter paper under a stack of paper towels for about 30 min. The dried nitrocellulose sheets sandwiched between the 3 mm filter paper, was wrapped in aluminium foil, sealed in a plastic bag and stored at -70°C. This is similar to the method used by Lin and Karamatsu (1983).

Detection of antigen-antibody complex using perioxidase-conjugated anti-human IgG.

The pretreated-nitrocellulose sheet, which contained antigen, was thawed briefly and soaked in 1% casein in PBS for 10 min at 37°C. This nitrocellulose sheet was incubated with the antibody IgG fraction(10mg/ml)(diluted to 1 in 250 with 1% casein in PBS) for 1 day at room temperature. The nitrocellulose sheet was then washed with 1% casein in PBS for 30 min (with 5 changes of buffer) and incubations continued with a 1/1000 dilution of perioxidase conjugated anti-human IgG in 1% casein in PBS overnight, at room temperature. The sheet was then washed for 30 min as described before. The sheet was briefly washed in 50mM Tris-HCl pH7.5 containing 0.9% NaCl and put into a freshly prepared solution of 0.06% (W/V) of 4-chloro-1-naphthol containing 0.01% H_2O_2 in 50mM Tris-HCl-saline, pH7.5. After about 5-10 min, dark blue or purple bands were seen. The reaction was terminated by washing with H_2O. The blots were stored and protected from light before taking a photograph. This procedure was adapted from the method of Mason and Sammons (1978).

5.2. Results.

5.2.1. Specificity and concentration of IgG solution from normal individuals and SLE patients.

Serum samples were obtained from clearly diagnosed SLE patients with autoantibodies of known specificity as determined by immunodiffusion and counterimmunoelctrophoresis. Serum samples were provided by Dr. P.Maddison and Dr.P.Skinner, the Royal National Hospital for Rheumatic Diseases, Bath. The sera were divided into 8 groups according to their ANA specificity and IgG fractions were prepared.

The specificity of some sera and all IgG fractions from both normal and SLE patients was again checked by immunodiffusion (Ouchterlony), radio-immunoassay, and enzyme-linked immunosorbant assay (ELISA). The results are summarized in Table 5.1. Some of these IgG fractions from SLE patients were prepared and identified by Mr.J.R. Dobson in this department. In all assays, normal serum or IgG fraction was used as a control.

The specificity of the ANA (both serum and IgG fraction) was checked by immunodiffusion analysis (Ouchterlony,1958). Human spleen extract gave precipitin lines with anti-Ro - and anti-Sm-antibodies while calf thymus extract was used with anti-La - and anti-n RNP -antibodies. Positive tests were compared to the known anti-serum which was used as a standard. All of serum and IgG fractions from normal individuals gave negative tests, no precipitin line with either calf thymus extract or human spleen extract. To identify antibody, radio-immunoassays were used and all of 5 samples (IgG fractions) gave positive tests.

The ELISA was also used to check the specificity of antiLa- antiRo- and antiDNA- antibodies. Both serum and IgG fraction were used and samples from normal individuals were used as controls. For antiRo, all sera samples gave positive tests while only

Table 5.1. Specificity and concentration of IgG solution from normal and SLE patients.

Name	Date	Hospital no.	Specificity	Ouchterlony test		ELISA test		Concentration(mg/ml)	
				CTE	HSE	serum	IgG	by A_{280}	Lowry method
I.									
1)W.Mullen	-	-	normal	negative	negative	negative	negative	5.52	6.5
2)K.Ounarom	-	-	"	"	"	"	"	N.D	4.4
3)W.Y.NG	-	-	"	"	"	"	"	"	6.0
4)M.El-Naggar	-	-	"	"	"	N.D	"	"	6.4
5)K.Thompson	-	-	"	"	"	"	"	"	7.3
6)Mc.Keowan	-	-	"	"	"	"	"	"	8.35
7)S.F.Chai	-	-	"	"	"	"	N.D	"	8.1
8)Mrs.M	-	-	"	"	"	"	"	"	9.85
9)Miss	-	-	"	"	"	"	"	"	8.55
10)B.Al-Ibrahim	-	-	"	"	"	"	"	"	8.0
II.									
1)Buffalo	-	-	antiLa	positive	N.D	positive(+3)	positive(+2)	3.67	3.8
2)S.Savory	-	-	"	"	"	N.D	" (+2)	6.29	N.D
3)Knowland	-	-	"	"	"	"	" (+2)	4.4	"
III.									
1)C.Frayne	8.7.80	-	antiRo	N.D	positive	positive(+3)	positive(+3)	4.02	4.3
2)N.Grillard	-		"	"	"	N.D	negative	5.42	N.D
3)D.Clement	21.8.81	RNHRD 42147	"	"	"	"	"	3.11	"
4)N.Grillard*	17.7.80	35234	"	"	"	positive(+1)	"	2.5	"
5)D.Clement*	11.4.80	28285	"	"	"	" (+2)	"	4.4	"
6)M.Miller	19.5.80	-	"		negative	" (+2)	"	N.D	3.8
7)Huggil	29.7.82	346680	"	"	positive	" (+3)	positive(+1)	"	4.05
8)E.Ashman	1.10.81	43029	"	"	"	" (+3)	" (+1)	"	5.85
9)T.Charles	2.2.82	43433	"	"	"	" (+1)	negative	"	6.45
10)Ravenhill	29.7.82	-	"	"	"	" (+3)	positive(+1)	"	5.9
11)N.Cuff	27.5.82	44613	"	"	"	" (+1)	negative	"	6.38
IV.									
1)M.Pryce	8.5.81	HGM	antiSm/Ro	N.D	positive	N.D	N.D	3.32	N.D

Table 5.1. (Continued)

Name	Date	Hospital no.	Specificity	Ouchterlony test		ELISA test		Concentration(mg/ml)	
				CTE	HSE	serum	IgG	by A_{280}	Lowry method
V.									
1)S.Davis	28.4.81	A 089 428C	antiRo/La	negative	positive	N.D	N.D	3.84	N.D
2)M.Jone	1.8.81	A 0314 6H	"	positive	"	"	"	5.24	5.25
3)S.Jone	21.5.81	Card 174	"	"	"	"	"	5.76	6.5
4)P.Burnett	-	-	"	"	"	"	"	13.10	N.D
5)M.Rydor	5.12.81	A 204 526D	"	negative	negative	"	"	6.12	"
VI.									
1)E.Swindell	-	A 201 047	antiRNP	positive	N.D	N.D	N.D	3.11	5.6*
2)M.Windsor	17.4.80	-	"	"	"	"	"	5.06	6.4*
3)R.Carter	2.3.81	-	"	"	"	"	"	6.51	N.D
4)Johanna	2.4.81	SMH 184 248	"	"	"	"	"	3.32	"
5)A.Key	25.4.81	Guys 501840D	"	"	"	"	"	6.29	"
6)M.Fry	13.11.81	--	"	"	"	"	"	2.09	"
7)E.Burness	5.8.80	Card(III)	"	"	"	"	"	4.54	"
VII.									
1)P.Summes	17.4.80	SM 550 577	antiSm/RNP	positive	positive	N.D	N.D	1.75	5.8*
VIII.									
1)P.Higginson	23.6.80	BRI	antiSm	N.D	positive	N.D	N.D	2.79	6.1*
2)V.Norton	1977	38867	"	"	"	"	"	0.87	2.85*
3)R.Hodges	3.2.81	SM 363298	"	"	"	"	"	2.79	N.D
IX.									
1)M.Holman	28.11.77	-	antiDNA	N.D	N.D	N.D	positive(+1)	10.50	9.3
2)P.Plummer	18.2.82	44039	"	"	"	"	" (+2)	3.6	4.6*
3)J.Hildea A	27.1.82	44292	"	"	"	"	" (+2)	1.65	2.45*
4)J.Hildea B			"	"	"	"	" (+1)	9.9	9.1
5)F.Jonas	28.8.81	435932	"	"	"	"	" (+3)	2.62	3.15

Note: For ELISA test; +3=highest absorbance and +1=absorbance was slightly higher than control.
* at concentration=IgG solution was concentrated before determined a concentration by Lowry method.
CTE=calf thymus extract; HSE=human spleen extract; N.D=not determined.

4 out of 9 IgG samples showed positive tests and three gave absorbances slightly higher than control while the rest were considered to give a negative test. These results were different from the immunodiffusion assay and this might be due to the antigen Ro which was used to coat the wells and bind to anti Ro-antibody in ELISA. Two out of five samples from SLE patients containing anti DNA-antibody showed low positive tests in the ELISA assay their absorbance was slightly higher than control. The reason of this result was not clearly understood but it is suggested that it might be related to the amount of specific ANA in each sample, the purity of antigen and the specificity of assay.

The concentration of IgG solution was determined by using either absorbance value at 280nm or Lowry's method and the results are shown in Table 5.1.

5.2.2. Effect of ANAs on TMV RNA translation in rabbit reticulocyte lysate cell-free systems

5.2.2.a. Effect of concentration of ANA and incubation time on TMV RNA translation.

The optimization of ANA concentration and incubation time were investigated. IgG solutions of various concentrations from normal and SLE patients were added to the reaction mixture before starting the incubation at 30°C. The incorporation of ^{35}S-Met into TMV protein was determined after 60 min of incubation. The incorporation of ^{35}S-Met decreased proportionately with increasing ANA concentration up to 0.2µg/µl (Fig.5.1.a.) At a concentration of 0.2µgANA/µl reaction mixture, the antiDNA (F.Jonas) and antiRo (C.Frayne) gave the highest inhibition, about 95%, while antiLa (Buffalo) gave only 36.6% inhibition and 6.5% inhibition was given by antiRo/La (S.Jone). For other ANA samples, a concentration of 0.2µg/µl reaction mixture was used to investigate the effect of ANA on TMV RNA translation.

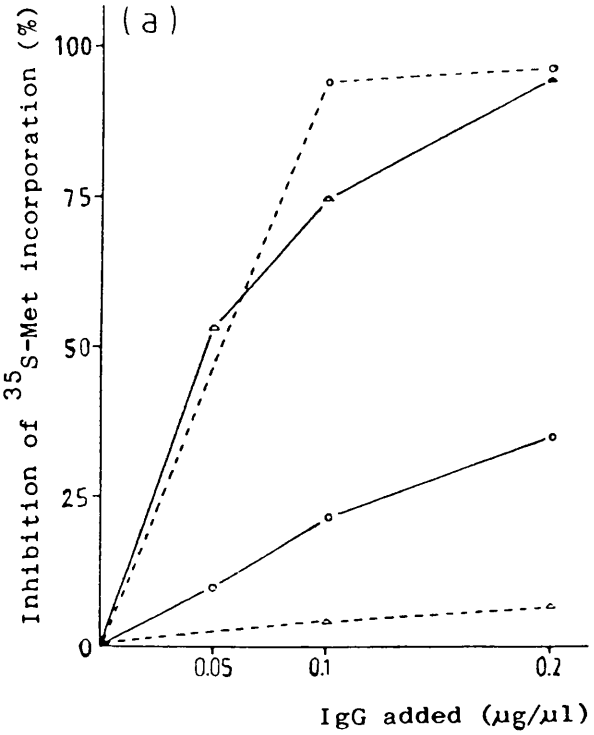
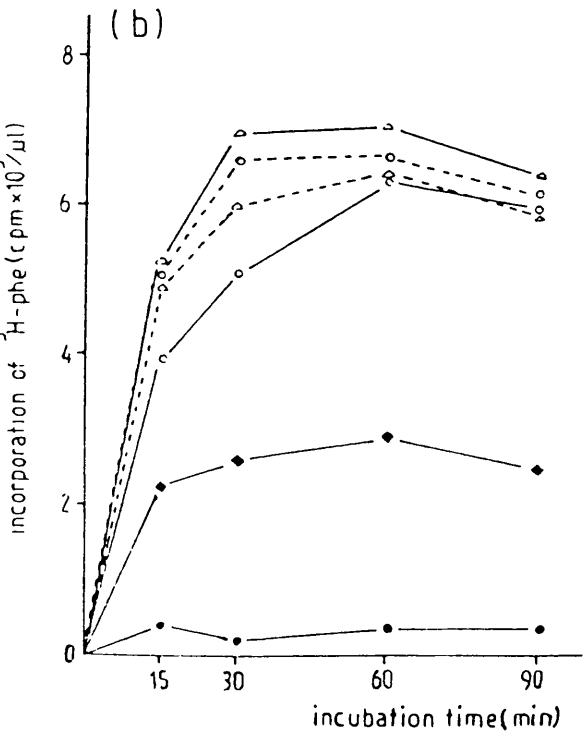
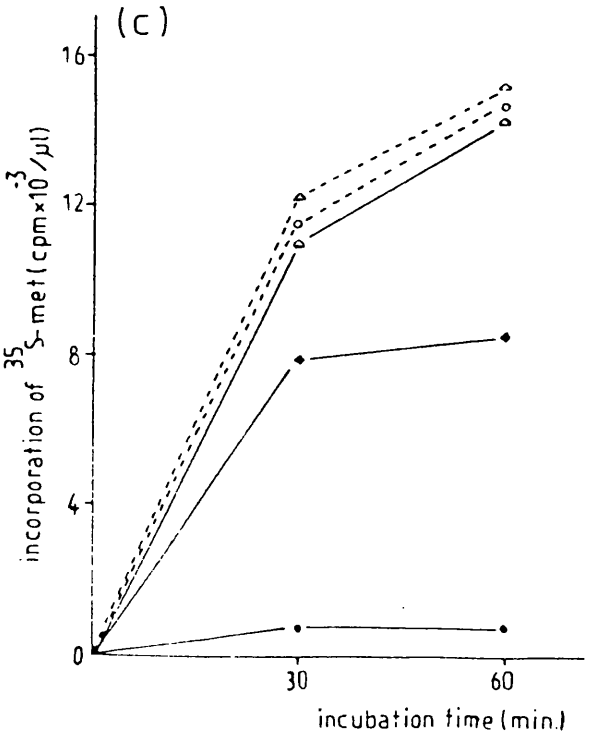


Fig. 5.1. The effect of ANAs on translation of TMV RNA in rabbit reticulocyte lysate cell-free system.

a) The effect of various concentrations of ANAs on TMV protein synthesis. Inhibition by ANAs was compared to normal IgG at 60 min incubation. antiDNA (Jonas)(o---o); antiRo(Frayne) (▲—▲); antiLa(Buffalo)(o—o); and antiRo/La(S.Jone)(▲---▲).





b) Effect of ANAs(0.2μg/μl) on translation of TMV RNA. The reaction mixture was incubated at 30°C with ^{3}H-Phe and all the points were the average of ^{3}H-Phe incorporation. The fluorogram of these translation products is shown in Fig.5.2.a. no IgG(▲---▲);normal IgG(Mullen)(▲—▲);antiRo(Frayne) (•—•);antiLa(Buffalo)(•—•);antiRNP(Burness)(o—o);and antiRo/La(M.Jone)(o---o).

c) Time-course experiment of TMV RNA directed incorporation of ^{35}S-Met in the absence and presence of IgG from normal and SLE patients at concn. of 0.2μg/μl. no IgG(▲---▲);normal IgG(Mullen)(o—▲);antiRo/La(Burnett)(o---o); antiLa(Buffalo)(•—•); and antiRo(Frayne)(•—•).

The radiolabelled amino acids ^{3}H-Phe and ^{35}S-Met, were used in translation of TMV RNA in the presence of ANAs and it was found that the inhibitory effect was not related to precursor used; for example anti Ro (Frayne) at a concentration of 0.2µg/µl reaction mixture gave 95.62% inhibition with ^{35}S-Met and 94.16% inhibition with ^{3}H-Phe (Fig 5.1.b. and 5.1.c.). Because a high specific activity was required for gel analysis, we have chosen ^{35}S-Met for studying the the effect of ANAs on TMV RNA translation.

The time course of incorporation was studied in this experiment. With ANA samples that have inhibitory effect on TMV RNA translation, lower incorporation of radioactivity into protein was shown immediately with only slightly increased incorporation up to 15 min. After 15 min of incubation, the incorporation tended to slow down and level off until 90 min (Fig. 5.1.b.). The incubation time of 30 and 60 min was used to test the rest of the ANA samples. Only the most inhibitory samples were repeated in time-course experiments.

5.2.2.b. Analysis of translation products on SDS-PAGE.

To determine which TMV protein was inhibited in the presence of ANA during translation of TMV RNA in reticulocyte lysate, the translation products were further analyzed on SDS-PAGE. The samples were loaded onto a gel with the same volume of reaction mixture in each well and the protein patterns were detected by fluorography.

The gel patterns of TMV protein were the same whether labelling was with ^{3}H-Phe or ^{35}S-Met (Fig.5.2.a.lane 2 and 3 and Fig. 5.2.b. lane 1 and 2, respectively). Two major proteins at M.Ws of 176K and 116K, as well as several protein bands at lower M.Ws of about 80-90K, 60-70K, 45-55K and 30-40K were shown. The protein pattern and

Fig. 5.2. Effect of ANAs on TMV RNA translation in rabbit reticulocyte lysate cell-free system. Fluorographic pattern of translation product separated on 5-15% SDS-PAGE after 90 min incubation with ^{3}H-Phe (a) or 60 min incubation with ^{35}S-Met (b-d).

(a)

lane 1: control without mRNA
" 2: added H_2O
" 3: " normal IgG(W.M)
" 4: " antiRo/La(Davis)
" 5: " antiLa(Buffalo)
" 6: " antiRo(Frayne)
" 7: " antiRNP(Burness)
" 8: " antiSm(Hodges)
" 9: " antiRo/La(M.Jone)
" 10: " standard proteins: ^{14}C-BSA; ^{14}C-RNase; and ^{14}C-trypsin inhibitor.

Exposure: 2,000-50,000 cpm; 20 days; -70°C.

(b)

lane 1: added normal IgG(W.M);0.2µg/µl
" 2: " " " (W.M);0.1 "
" 3: " antiLa(Buffalo);0 2 "
" 4: " " (Buffalo);0.1 "
" 5: " " (Buffalo);0.05 "
" 6: " antiRo(Frayne);0.1 "
" 7: " " (Frayne);0.05 "
" 8: " antiRo/La(S.Jone);0.2"
" 9: " antiRo/La(Burnett);0.2"

Exposure: 21,000-100,000 cpm; 2 days; -70°C.

(c)

Normal IgG and ANAs were added to the reaction mixture at concn. of 0.2 µg/µl.

lane 1: added normal IgG(W.M)
" 2: " antiRo(Clement)
" 3: " antiSm/Ro(Pryce)
" 4: " antiRNP(Swindell)
" 5: " antiRNP(Carter)
" 6: " normal IgG(W.M)
" 7: " antiRNP(Windsor)
" 8: " antiSm/RNP(Summes)
" 9: " antiDNA(Holman)
" 10: " antiDNA(Hildea A)

Exposure: 200,000-300,000 cpm; 1day : -70°C.

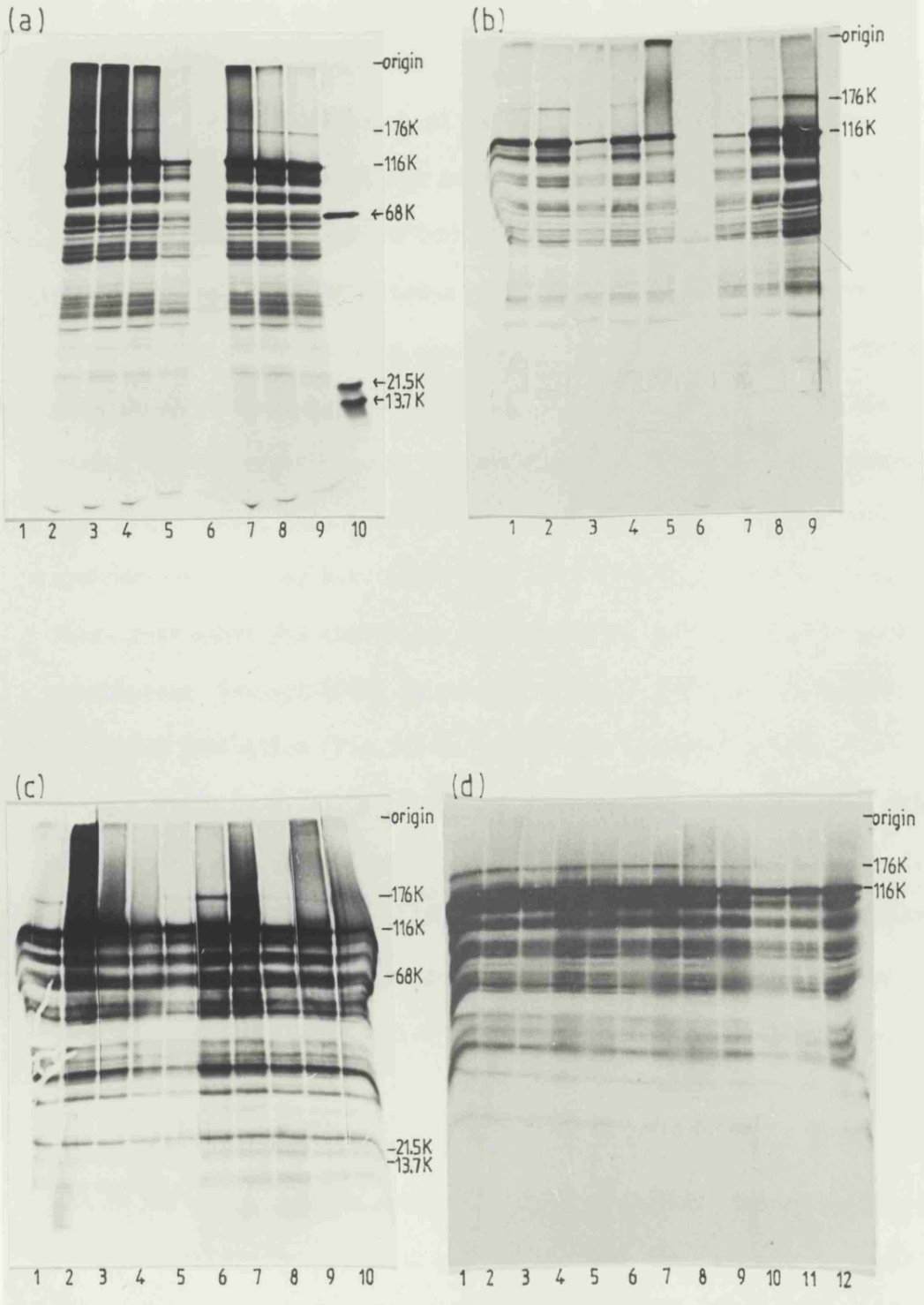
(d)

Normal IgG and ANAs were added to the reaction mixture at concn. of 0.2 µg/µl.

lane 1: added normal IgG(K.O)
" 2: " antiRo(Miller)
" 3: " antiRo(Ashman)
" 4: " antiRo(Ravenhill)
" 5: " antiRo(Cuff)
" 6: " antiRo(Gillard)
" 7: " antiRo(Clement)
" 8: " normal IgG(NG)
" 9: " antiRo/La(M.Jone)
" 10: " antiRo/La(S.Jone)
" 11: " antiRo/La(Rydor)
" 12: " antiRo/La(S.Jone)

Exposure: 100,000- 300,000 cpm; 2 days; -70°C

Fig. 5.2


(a)
origin
176K
116K
68K
21.5K
13.7K
1 2 3 4 5 6 7 8 9 10
(b)
origin
176K
116K
1 2 3 4 5 6 7 8 9
(c)
origin
176K
116K
68K
21.5K
13.7K
1 2 3 4 5 6 7 8 9 10
(d)
origin
176K
116K
1 2 3 4 5 6 7 8 9 10 11 12

the amount of protein synthesized were the same when PBS or normal IgG solutions were added to the reaction mixture (Fig.5.2.a. lane 2 and 3), therefore, we used the translation product in the presence of normal IgG as a control.

From fluorograms of SDS-PAGE of TMV protein synthesized in the presence of normal IgG and ANAs(Fig.5.2.a,b,c, and d) it was found that the inhibitory effect of ANAs was not restricted to any specific protein. All types of proteins were synthesized in smaller amounts when the inhibition by ANAs was shown. With antiRo (Frayne) which gave about 95% inhibition, there was no protein synthesis. This result was the same as using translation products without exogeneous mRNA (Fig. 5.2.a, lane 6 and lane 1, respectively). Protein was synthesized at lower M.W. (less than 65K) in sample antiRo (Frayne), which gave about 74% inhibition and nearly all sizes of protein were synthesized (except 176K) in sample antiRo (Frayne) which gave about 52% inhibition (Fig.5.2.b. lane 6 and 7,respectively). Because protein at M.W.176K was synthesized in smaller amount than other proteins at M.W. of 116K or less than 116K, it seemed to be most affected by ANAs which gave an inhibitory effect on TMV RNA translation. With antiLa (Buffalo) which gave about 36% inhibition, there was no synthesized protein at M.W. of 176K (Fig.5.2.a. lane 5 and Fig. 5.2.b. lane 3).

The gel pattern was the same as ANA-free controls when there was no effect on protein synthesis, such as antiRNP (Burness), antiSm (Hodges), antiRo/La (Burnett) and antiRo (Miller, Ashman, Ravenhill) (Fig.5.2.a.lane 7 and 8, Fig.5.2.b. lane 9 and Fig.5.2.d. lane 2-4, respectively).

Table 5.2. Percent inhibition of ANAs on TMV protein synthesis in rabbit reticulocyte lysate cell-free system.

Specificity / Name	Amount of IgG (µg/µl)	% Inhibition at 30 min	% Inhibition at 60 min
I. Normal			
1. W.Mullen	0.2	-26.79±10.40	-15.63±4.60
2. K.Ounarom	0.2	-0.49±18.70	5.20±9.96
3. W.Y.NG	0.2	-22.10±26.15	-6.97±9.89
4. M.El-Naggar	0.2	5.96	-18.70
5. K.Thompson	0.2	-17.90±14.70	-6.60±3.80
6. Mc.Keowan	0.2	-22.55±5.86	-18.84±8.60
7. S.F.Chai	0.2	-8.40±4.67	9.98±10.50
8. Mrs.M	0.2	-19.95±4.45	6.59±6.36
9. Miss	0.2	-12.75±2.33	12.35±11.50
10. B.Al-Ibrahim	0.2	0.12	2.86
		-12.48±11.27	-2.98±11.94
II. AntiLa			
1. Buffalo	0.2	17.88±22.08	36.60±8.39
	0.1	7.11±10.32	20.80±0.71
	0.05	21.0	9.7
2. S.Savory	0.2	N.D	-5.7
3. Knowland	0.2	N.D	-13.5
III. AntiRo			
1. C.Frayne	0.2	95.02±3.04	95.62±2.07
	0.1	78.5	74.0
	0.05	43.3	52.3
2. N.Gillard	0.2	6.90±5.32	11.40±13.56
3. D.Clement	0.2	0.83±13.34	0.90±14.4
4. N.Gillard*	0.2	-7.65±3.32	7.85±7.0
5. D.Clement*	0.2	-4.02±5.49	2.35±1.9
6. M.Miller	0.2	-11.80±2.26	-4.15±7.99
7. Huggill	0.2	-9.8	3.3
8. E.Ashman	0.2	3.3	5.3
9. T.Charles	0.2	2.0	5.6
10. Ravenhill	0.2	5.80±7.49	11.18±4.41
11. N.Cuff	0.2	13.80±6.08	26.15±1.34
IV. AntiSm/Ro			
1. M.Pryce	0.2	-11.50	-16.10

Table 5.2.(continued)

Specificity / Name	Amount of IgG (μg/μl)	% Inhibition at 30 min	% Inhibition at 60 min
V. AntiRo/La			
1. S.Davis	0.2	6.73	2.78
2. M.Jone	0.2	20.20±10.6	10.35±7.42
3. S.Jone	0.2	7.56±10.87	10.90±2.75
4. P.Burnett	0.2	N.D	-5.70
5. M.Rydor	0.2	12.90	-8.50
VI. AntiRNP			
1. E.Swindell	0.2	1.93±11.1	8.30±8.11
2. M.Windsor	0.2	4.79±6.65	4.45±15.06
3. R.Carter	0.2	-6.6	5.3
4. Johanna	0.2	-22.5	-6.8
5. A.Key	0.2	10.9	-0.89
6. M.Fry	0.2	6.1	4.3
7. E.Burness	0.2	-16.1	-23.4
VII. AntiSm/RNP			
1. P.Summes	0.2	10.85±9.54	22.25±6.69
VIII. AntiSm			
1. P.Higginson	0.2	12.95±3.75	16.75±2.05
2. V.Norton	0.2	16.55±0.07	30.85±10.43
3. R.Hodges	0.2	-19.4	9.0
IX. AntiDNA			
1. M.Holman	0.2	20.47±9.50	19.61±12.64
2. P.Plummer	0.2	-7.17±22.6	6.56±10.86
3. J.Hildea A	0.2	32.85±3.88	39.35±4.45
4. J.Hildea B	0.2	-3.8	-6.7
5. F.Jonas	0.2	95.11±1.78	96.26±1.21
	0.1	95.1	95.9

Note: The negative values of % inhibition represent stimulation of incorporation of radioactivity.

N.D = not determined.

5.2.2.c. The inhibitory effect of ANAs on translation of TMV RNA.

36 ANA samples from 8 groups for SLE patients and 10 samples of IgG fractions from normal human serum were used to study their effects on TMV RNA translation. The inhibition index of ANAs was quoted as the percent inhibition compared to normal IgG at 30 and 60 min of incubation while for normal IgG, this value was compared to just PBS or H_2O in the reaction mixture.

The average values of percent inhibitions of ANAs relative to normal IgG and percentage inhibitions of normal IgGs were calculated and are summarized in Table 5.2 and Fig.5.3.

Normal IgG samples showed a slight effect on TMV RNA translation in terms of radioactivity incorporation when compared to controls without any IgG solution in the reaction mixture. The mean value of protein inhibition was $-12.48 \pm 11.27\%$ and $-2.98 \pm 11.94\%$ at 30 and 60 min of incubation, respectively, representing a slight increase in incorporation.

The inhibition of TMV RNA translation did not relate to any individual type of ANA; for example, with antiRo, 4 samples out of 9 gave inhibition higher than 10% and only one of these samples gave very high inhibition, about 95%. For antiSm and antiDNA most of the samples had an effect on TMV RNA translation and the inhibition varied from 6% to 96%. Only one sample of antiLa gave any inhibition of approximately 36%. There was no inhibition or very low inhibition of TMV protein synthesis (3% to 10%) by anti Ro/La and antiRNP groups.

5.2.2.d. Effect of salt concentration on TMV RNA translation.

All IgG samples of normal individuals and SLE patients were in PBS solution and were diluted with H_2O to the required con-

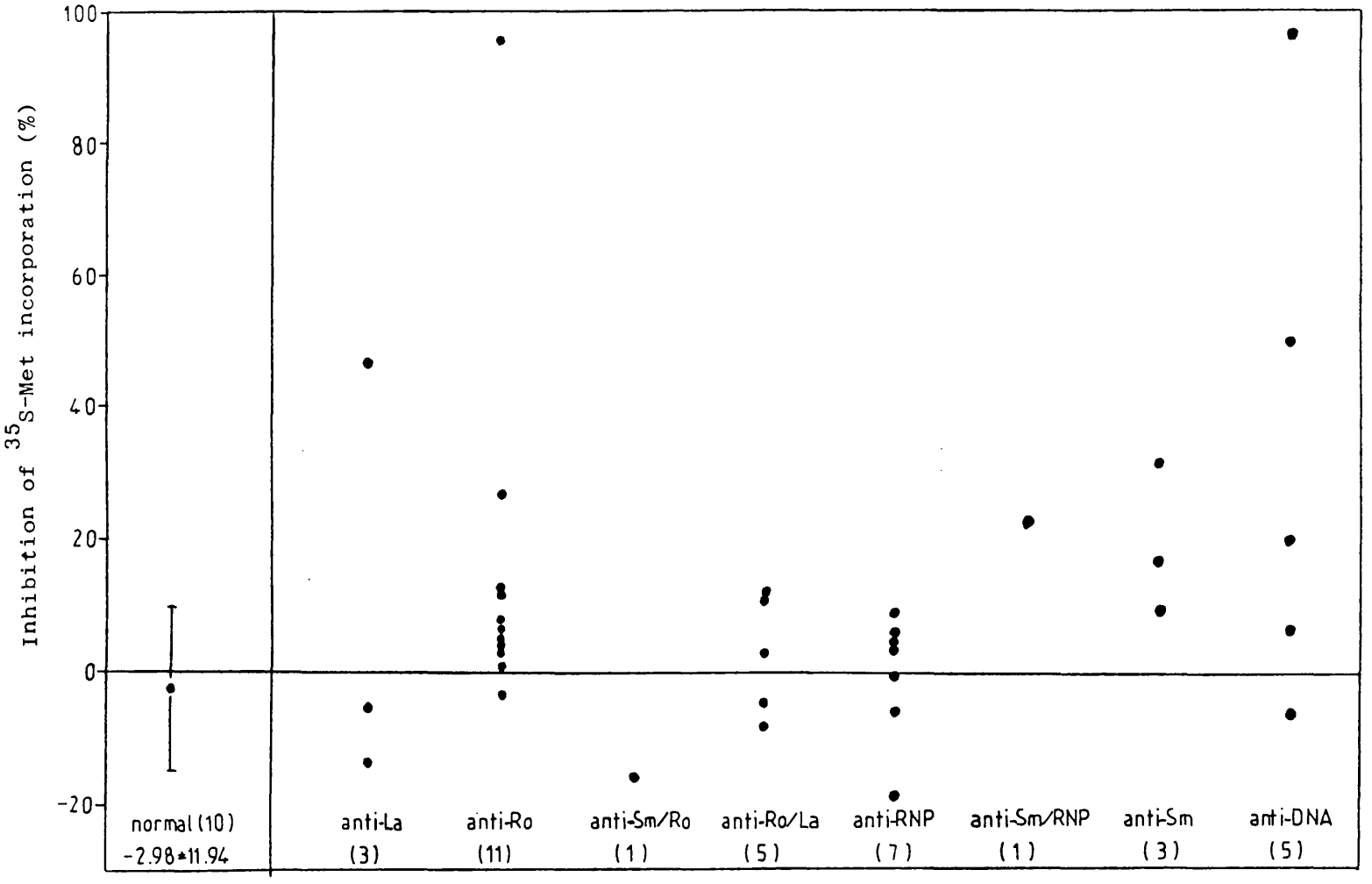


Fig. 5.3. Comparison of inhibition by ANAs from different SLE subgroups in translation of TMV RNA in reticulocyte lysate cell-free system. The value of percent inhibition of ANAs was calculated at 60 min of incubation. The mean value with standard deviation was shown for normal when normal IgG solution (10 samples) was added to reaction mixture.

Table 5.3. Effect of PBS on translation of TMV RNA and comparison of inhibitory effect of ANAs on conductivity.

Specificity / Name	% Inhibition at 60 min	Conductivity (μmho)
10.5 μM PBS	0.56	3.8
38.0 μM PBS	-3.60	7.2
84.0 μM PBS	16.60	12.2
210.0 μM PBS	54.3	27.5
420.0 μM PBS	90.6	52.0
I. Normal		
1. W.Mullen	-15.63±4.6	4.08±1.09
2. K.Ounarom	5.20±9.96	4.3
3. W.Y.NG	-6.97±9.89	4.8
II. AntiLa		
1. Buffalo	36.60±8.39	2.44±0.06
III. AntiRo		
1. C.Frayne	95.62±2.07	5.33±2.74
2. N.Grillard	11.40±13.56	4.5
3. D.Clement	0.90±14.4	5.0
IV. AntiSm/Ro		
1. M.Pryce	-16.10	9.0
V. AntiRo/La		
1. S.Jone	10.90±2.75	4.65
2. M.Jone	10.35±7.42	5.0
3. M.Rydor	-8.5	4.5
VI. AntiRNP		
1. E.Swindell	8.30±8.11	4.78±1.45
2. M.Windsor	4.45±15.06	4.98±0.6
3. R.Carter	5.3	4.25
4. Johanna	-6.8	5.0
5. A.Key	-0.89	4.8
6. M.Fry	4.3	6.5
VII. AntiSm/RNP		
1. P.Summes	22.25± 6.69	8.95±3.46
VIII. AntiSm		
1. P.Higginson	16.75±2.05	7.7
2. V.Norton	30.85±10.43	6.0
IX. AntiDNA		
1. M.Holman	19.61±12.64	3.88±0.03
2. P.Plummer	6.56±10.86	4.48±0.74
3. J.Hildea A	39.35±4.45	7.95±0.64
4. J.Hildea B	-6.7	5.5
5. F.Jonas	96.26±1.21	6.5

Note: The negative values of % inhibition represent stimulation of incorporation of radioactivity.

centration (1.1µg/µl). The PBS solution at a concentration of 0.15M, contained NaCl, KCl, KH_2PO_4 and Na_2HPO_4. Translation of mRNA in rabbit reticulocyte lysate cell-free systems is dependent on salt concentration. At high concentrations of Mg^{2+} salts, K^+ salts, or Cl^- salts, the radioactivity incorporation was significantly reduced as shown by Weber et al. (1977), Suzuki (1977 and 1981) and Bhargava (1983).

We have proved that the inhibitory effect of some ANA samples on TMV translation in this cell-free system was not a result of salt concentration by testing the effect of PBS at various concentration on TMV protein synthesis and comparing the present inhibition to amount of salt (conductivity value). The conductivity of most of the IgG solutions from normal and SLE patient which were used to test the inhibitory effect on TMV RNA translation was also determined. These conductivity values of the IgG samples and PBS samples were compared to their percent inhibition as presented in Table 5.3 and Fig. 5.4.a. On varying the PBS concentration (10.5µM to 420µM), the incorporation of ^{35}S-Met was decreased at higher concentration of PBS. The percent inhibition with these PBS samples was compared to the effect of adding H_2O to the reaction mixture. At low concentration of PBS (10.5µM - 38µM) or at conductivity less than 8µmho, there was no effect on TMV RNA translation (-3.6 to 0.56% inhibition). The inhibition increased to 90.6% when PBS was added at a concentration of 420µM, or conductivity of 52µmho, in the reaction mixture. Therefore, PBS concentration did have an inhibitory effect on TMV RNA translation (Fig. 5.4.a.) As shown in(Fig.5.4.aand Table 5.3, most of the IgG samples from both normal and SLE patient had conductivity values less than 9.0µmho. The ANAs which gave high percentage inhibition, such as antiRo (C.Frayne) and antiDNA (F.Jonas) had conductivity of 5.33 and 6.5 µmho, respectively. Therefore, the inhibitory

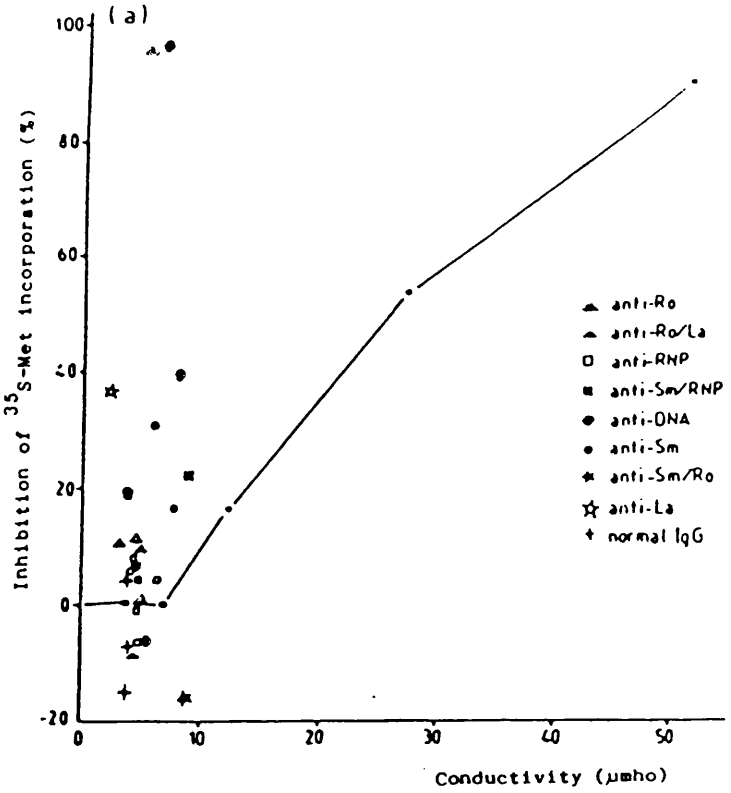


Fig. 5.4. a) Effect of PBS on translation of TMV RNA in reticulocyte lysate: Comparison of percentage inhibition with conductivity of IgG solution. IgG from both normals and SLE patients(at concn. of0.2 μg/μl) and various concentrations of PBS were added to the reaction mixture and incorporation of radioactivity was determined at 60 min incubation. The inhibition was dependent on PBS concentration, shown as conductivity value(•—•). The conductivity of ANA samples was determined and related to their percentage inhibitions.

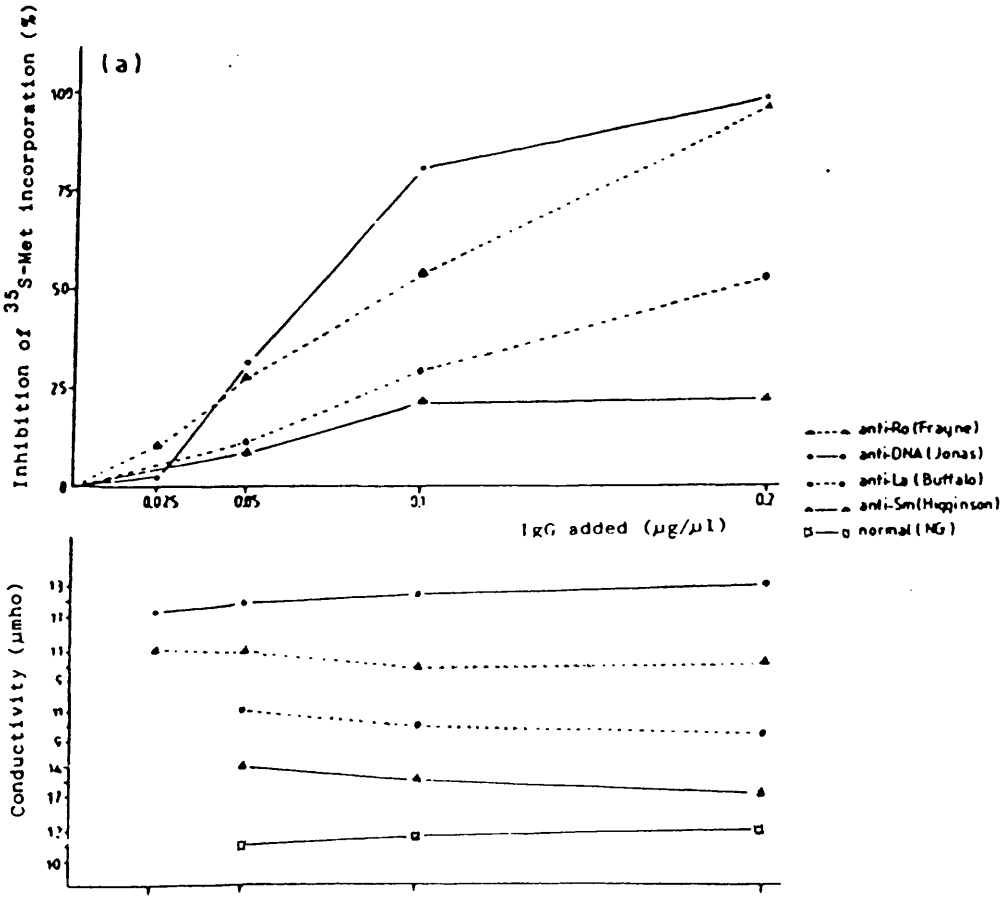
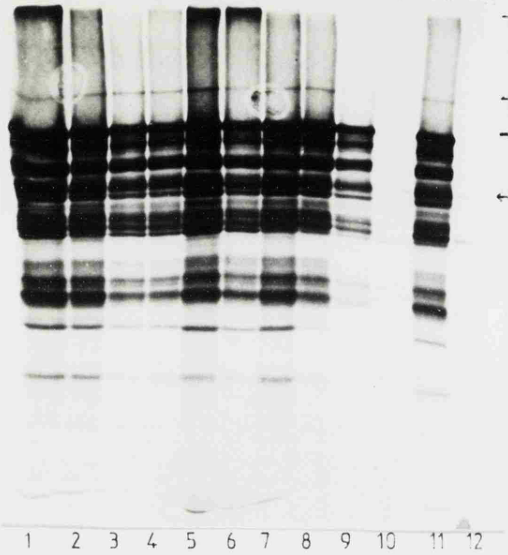


Fig. 5.5. TMV protein synthesized in reticulocyte lysate in the presence of different concentrations of ANAs and normal IgG.

a) Comparison of percent inhibition by different concentrations of ANAs to conductivity.

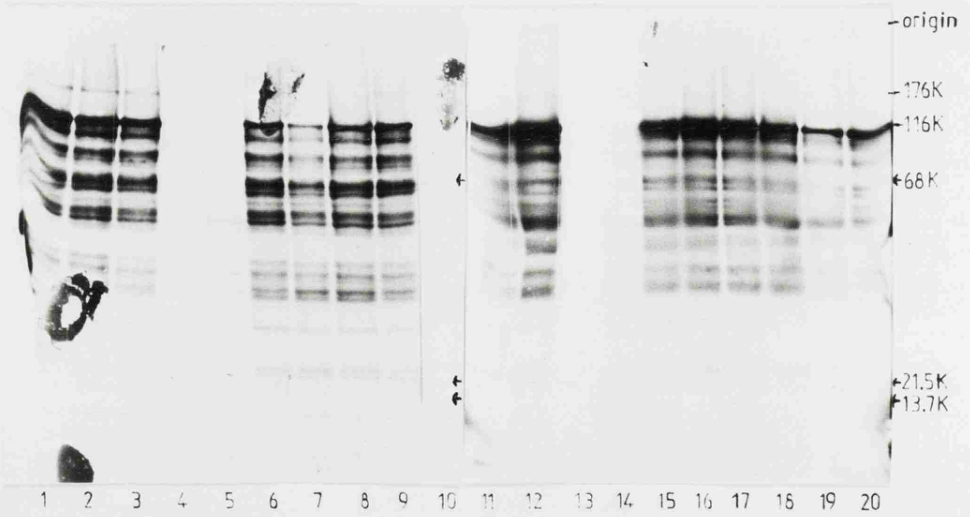
Fig. 5.4. b)Effect of salt(PBS and phosphate buffer), protein and DNA on TMV protein synthesis. Fluorogram of SDS-PAGE of ^{35}S-Met labelled protein is shown.



lane 1: added H_2O
" 2: " PBS, at 38μM
" 3: " DNA, at 0.003A_{260}
" 4: " DNA, at 0.0015A_{260}
" 5: " normal IgG(W.M)
" 6: " BSA, at 0.2μg/μl
" 7: " normal IgG(K.O)
" 8: " PBS, at 84μM
" 9: " PBS, at 210μM
" 10: " PBS, at 420μM
" 11: " phosphate buffer, at 3.63mM
" 12: " phosphate buffer, at 9.09mM

Exposure: 50,000-600,000 cpm; 2 days; -70°C.

Fig. 5.5. b)Fluorographic image of labelled TMV protein synthesized in the presence of ANA and normal IgG from (a).



lane 1: added H_2O
" 2: " normal IgG(NG), 0.2μg/μl
" 3: " " " (NG), 0.1 "
" 4: " antiRo(Frayne), 0.2 "
" 5: " " (Frayne), 0.1 "
" 6: " " (Frayne), 0.05 "
" 7: " antiLa(Buffalo), 0.2 "
" 8: " " (Buffalo), 0.1 "
" 9: " " (Buffalo), 0.05"
" 10: " standard proteins
lane 11: added normal IgG(NG), 0.1μg/μl
" 12: " " " (NG), 0.05 "
" 13: " antiDNA(Jonas), 0.2 "
" 14: " " (Jonas), 0.1 "
" 15: " " (Jonas), 0.05 "
" 16: " " (Jonas), 0.025"
" 17: " antiSm(Higginson), 0.2"
" 18: " " (Higginson), 0.1"
" 19: " " (Higginson), 0.05"
" 20: " antiRo(Frayne), 0.025 "

Exposure: 8,000-330,000 cpm; 2 days; -70°C

effect of ANAs on TMV translation should not be due to PBS concentration.

Phosphate buffer was also tested for effects on TMV RNA translation and it was found that the inhibitory effect depended on the concentration of phosphate buffer. At 1.81mM of phosphate buffer, there was no effect on protein synthesis and the percent inhibition was increased to approximately 16% and 85% at concentrations of 3.63mM and 9.09mM in the reaction mixture, respectively. Calf thymus DNA and BSA at concentrations of 0.003 A_{260} units/µl and 0.2µg/µl of reaction mixture, respectively, showed no effect on TMV RNA translation in reticulocyte lysate.

A fluorogram of SDS-PAGE of these translation products is shown in Fig.5.4.b. At high percentage inhibition, due to 420µM PBS or 9.09mM phosphate buffer, there was no protein synthesis (Fig.5.4.b. lane 10 and 12, respectively). The protein was synthesized at low M.W. where there was lower inhibition (Fig.5.4.b. lane 9 and 11). The gel patterns were the same as controls in samples which gave no inhibitory effect on TMV RNA translation (Fig.5.4.b. lane 2-4,6 and 8).

The inhibitory effect of ANAs on TMV RNA translation was confirmed to depend on the amount of IgG added, and to be independent of PBS concentration by the experiment using IgG solution from both normal and SLE patient diluted to optimal concentration with PBS solution. All of the IgG samples had different amounts of IgG but the amounts of PBS were nearly the same as shown by conductivity value (Fig.5.5.a.). For normal IgG at different concentrations in the reaction mixture (0.02 to 0.2µg/µl), the incorporation of ^{35}S-Met is slightly changed so we used the mean value of these incorporations of ^{35}S-Met to calculate the percent inhibition by ANA samples.

hen adding ANAs at various concentrations into reaction mixture, all

the ANA samples antiRo(Frayne), antiDNA (Jonas), antiLa (Buffalo) and antiSm (Higginson) showed that the percentage inhibition was dependent on the concentration of IgG (Fig.5.5.a.).

The translation products were further analyzed on SDS-PAGE and the fluorogram is shown in Fig 5.5.b. The amount of protein synthesized was related to percent inhibition and all sizes of proteins were synthesized in reduced amount. There was no protein synthesis at high percent inhibition (Fig. 5.5.b, lane 4 and 13). At low percent inhibition, the protein band at M.W. of 176K was not seen (Fig.5.5.b, lane 6, 7, and 15).

5.2.2.e. Comparison of inhibition of TMV RNA translation by ANAs and other protein synthesis inhibitors.

The protein inhibitors aurin tricarboxylic acid and cycloheximide were used in this experiment. Aurin tricarboxylic acid is known to inhibit initiation of protein synthesis in cell-free systems by preventing attachment of mRNA to ribosomes and subunits of both pro-and eukaryotes (Mathews,1971). Cycloheximide inhibits the release of tRNA and the movement of the ribosome on mRNA template, therefore, the translation step is stopped (Chan et al.,1973). Bathurst and Smith (1982) have used these protein synthesis inhibitors to compare the mechanism of inhibitory action on protein synthesis by small nuclear RNAs. The mode of action of ANAs on TMV RNA translation was investigated by comparing their action in a time-course experiment with that of the known protein synthesis inhibitors, cycloheximide and aurin tricarboxylic acid. After 10 min of incubation, ANAs and protein synthesis inhibitors were added to the reaction mixture to give a final concentration of 0.2μg/μl and 50μM, respectively and the kinetics of inhibition was determined until 60 min of incubation. The result is shown in Fig.5.6.a. and 5.6.b.

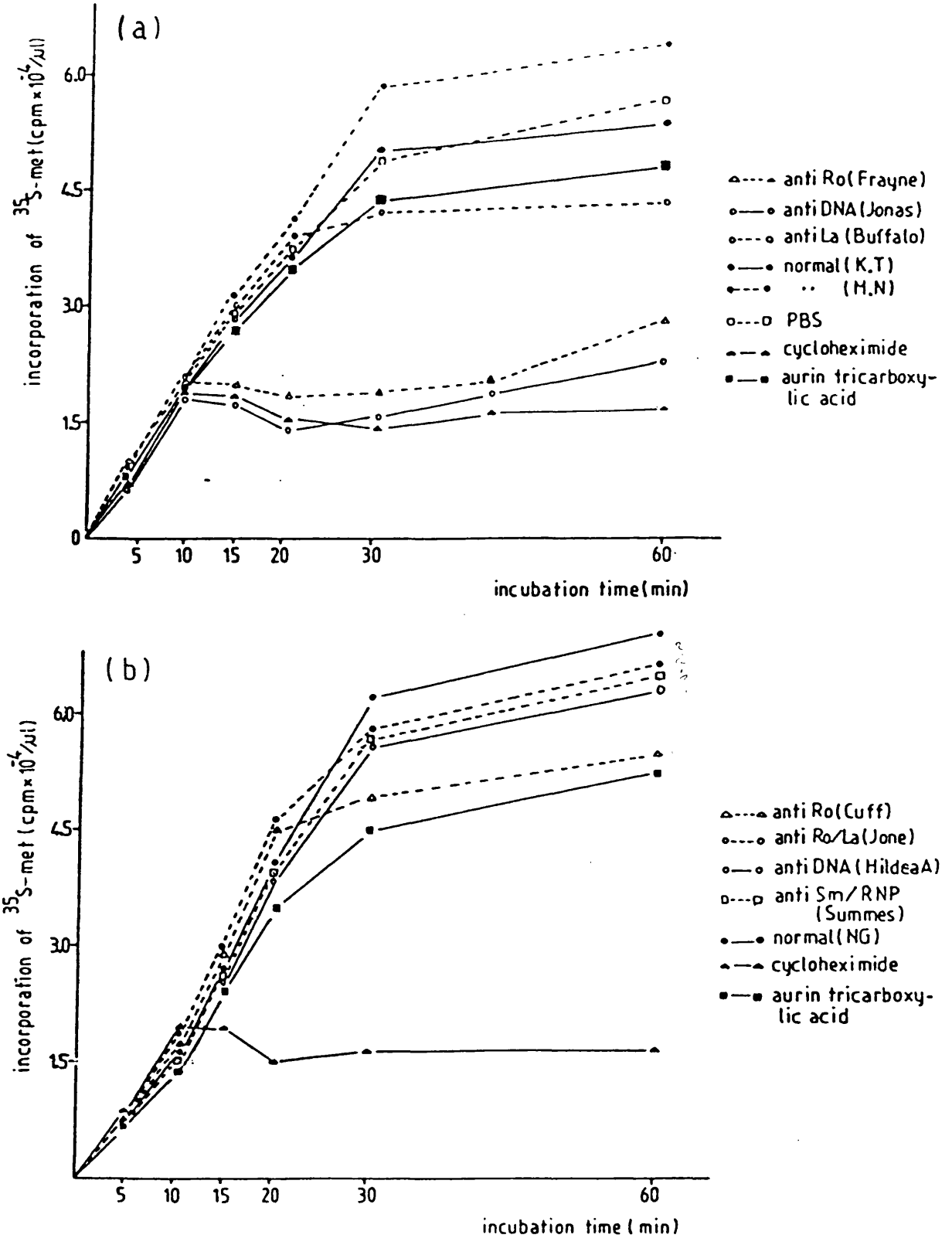
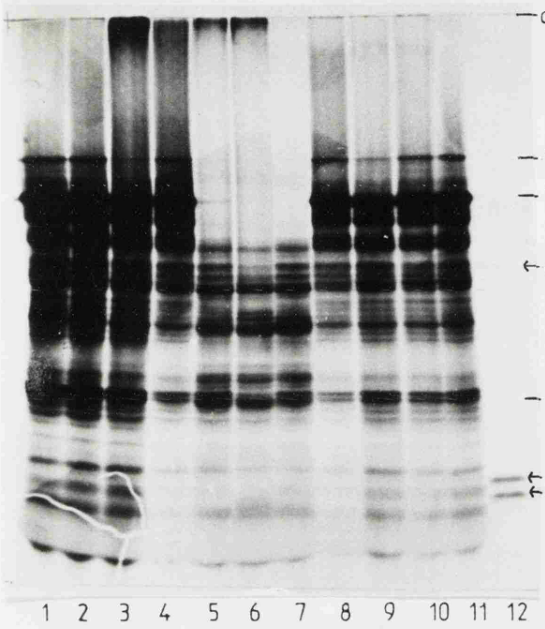


Fig. 5.6. Comparison of inhibitory effect of ANAs, cycloheximide and aurin tricarboxylic acid on translation of TMV RNA in a rabbit reticulocyte lysate cell-free system.

a and b) Time-course experiment: after 10 min of incubation, ANAs, PBS or normal IgG, and protein inhibitors (cycloheximide and aurin tricarboxylic acid) were added to reaction mixtures at a final concentration of 0.2µg/µl and 50µM; respectively.

Fig. 5.6. c and d) The translation products (from a and b) were taken for analysis by SDS-PAGE and fluorographic patterns are shown.

(c)



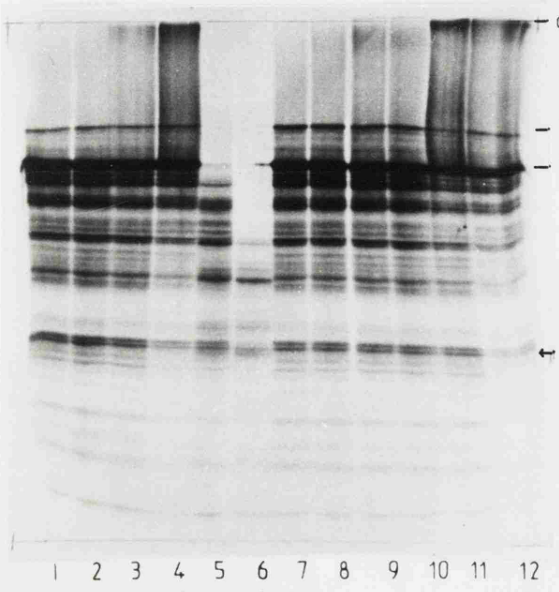
lane 1-6: the same volume of reaction mixture.

" 7-10: the same amount of radioactivity.

" 1: added PBS
" 2: " normal IgG(M.N)
" 3: " antiLa (Buffalo)
" 4: " aurin tricarboxylic acid (ATA).
" 5: " antiRo (Frayne)
" 6: " cycloheximide
" 7: " antiRo (Frayne)
" 8: " ATA
" 9: " antiLa (Buffalo)
" 10: " normal IgG(K.T)
" 11: " normal IgG(M.N)
" 12: " standard proteins

Exposure: 67,000-270,000 cpm; 2 days; -70°C.

(d)



lane 1-12: the same volume of reaction mixture.

" 1: added PBS
" 2: " normal IgG(K.T)
" 3: " antiSm (Higginson)
" 4: " ATA
" 5: " antiDNA (Jonas)
" 6: " cycloheximide
" 7: " normal IgG(NG)
" 8: " antiRo/La (Jone)
" 9: " antiSm/RNP(Summes)
" 10: " antiDNA (HildeaA)
" 11: " antiRo (Cuff).
" 12: " ATA

Exposure: 50,000-200,000 cpm; 1 day; -70°C.

The effect of antiRo (Frayne) and antiDNA (Jonas) was similar to cycloheximide, the incorporation of ^{35}S-Met into protein was immediately inhibited. This inhibition occured at the level of elongation, whereas the inhibition by aurin tricarboxylic acid was at the initiation step. The results demonstrate that antiLa (Buffalo), antiRo (Cuff), antiDNA (Hilder A.), antiSm/RNP(Summes) and antiRo/La (S.Jone) gave a curve which mimicked aurin tricarboxylic acid in which the inhibition of incorporation of radioactivity occured after a lag period of 15 to 20 min of incubation (Fig 5.6.a. and 5.6.b.).

The results were confirmed by analysis of translation products on SDS-PAGE and the fluorograms are shown in Fig.5.6.c. and 5.6.d. When inhibition was at the elongation step, protein at M.W. higher than 70K was not synthesized and low M.W. protein was synthesized in smaller amounts in the sample which gave inhibition at initiation step (Fig.5.6.c. lane 5-7, 5.6.d. lane 5 and 6 and Fig. 5.6.c. lane 4 and 8, 5.6.d. lane 3,4, and 12,.respectively). For antiLa (Buffalo), the protein bands at M.W. less than 32K were more intense than with aurin tricarboxylic acid, but less protein at M.W. of 176K was synthesized. (Fig. 5.6.c. lane 3, 4, 8 and 9).

5.2.2.f. Effect of specific antiRo-and antiLa -antibodies on TMV RNA translation.

Specific antiRo- and antiLa- antibodies were purified by using antigen Ro-and antigen La-Sepharose 4B affinity chromatography, respectively.

IgG solution of antiRo (C.Frayne, about 2mg), was applied to antigen Ro-Sepharose 4B column. The unbound fraction was washed with PBS and collected into 4 fractions and the bound fraction was eluted with guanidine-HCl buffer, collected into 3 fractions and immediately dialysed against PBS overnight. The specificity of

antiRo was tested by ELISA and it was found that both bound and unbound fractions had lost their Ro specificity (Fig. 5.7.a.). These fractions were tested for the inhibitory effect on TMV RNA translation in rabbit reticulocyte lysate and it was found that there was a low inhibition, about 6-18% for the unbound fraction number 1 and 2 at the concentration of 0.8-2.2µg in 11µl reaction mixture and this inhibition was increased to 11-32% for bound fraction at concentrations of 0.38-1.04µg in 11µl of reaction mixture. Before passing through the column this antiRo (C.Frayne) gave 93-94% inhibition and 74% inhibition at the concentrations of 2.2µg and 1.1µg/11µl reaction mixture, respectively.

Specific antiLa was purified from antiLa (Buffalo) IgG solution by affinity chromatography, the same procedure as antiRo. The unbound fraction (no 1-2) and bound fraction (no 1-3)were tested for antiLa activity by ELISA and it was found that both bound and unbound fractions still had antiLa specificity but the absorbance at 405 nm was less than the initial antiLa (Buffalo) IgG preparation (Fig.5.7.b.). This might be due to the overloading of the column which meant that the column could not bind all of the antiLa. On testing for inhibitory effect on TMV RNA translation in rabbit reticulocyte lysate, the unbound fraction had low percent inhibition (about 6%) at a concentration of 2.2µg in 11µl reaction mixture while the bound fraction gave higher inhibition, 16-18% at concentrations of 0.22-0.37µg in 11µl reaction mixture. The inhibition was higher than antiLa (Buffalo) before passing through the column, which had 38% inhibition at the concentration of 2.2µg in 11µl reaction mixture. This result showed that the inhibitory effect of ANAs on TMV RNA translation might be related to specific antiLa. In contrast, the unbound fraction, which had nearly the same specificity (by ELISA

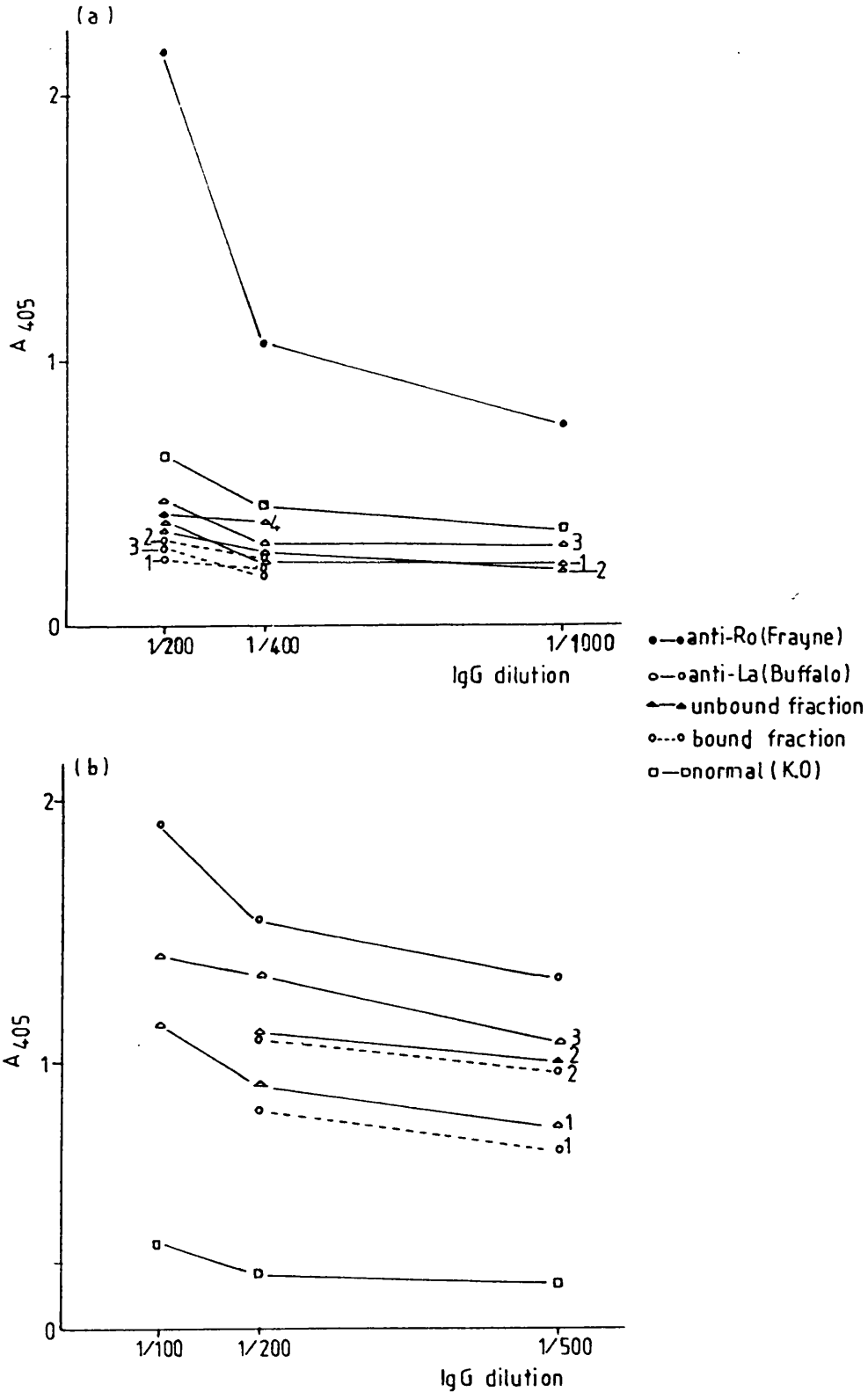


Fig. 5.7. a) ELISA test for antiRo in unbound(1-4) and bound(1-3) fractions of antiRo after passing through affinity chromatography.

b) ELISA test for antiLa in unbound(1-3) and bound(1-2) fractions of antiLa after passing through affinity chromatography.

All IgG preparations had the same concentration (10mg/ml) before making the dilution of 1/100-1/1000.

Table 5.4. Percent inhibition of globin synthesis by ANAs in reticulocyte lysate.

Specificity / Name	Amount of IgG (μg/μl)	% Inhibition at 30 min	% Inhibition at 60 min
I. Normal			
1. K.Ounarom	0.2	10.26	12.14
2. W.Y.NG	0.2	-34.22	-8.97
3. M.El-Naggar	0.2	7.08	-7.4
4. K.Thompson	0.2	2.37	-23.08
5. Mc.Keowan	0.2	-12.43	-18.19
6. S.F.Chai	0.2	8.28	14.99
7. Mrs.M	0.2	5.28	8.17
8. Miss	0.2	-7.76	-7.10
9. B.Al-Ibrahim	0.2	3.68	-4.50
		-0.22±14.39	-3.77±13.14
II. AntiLa			
1. Buffalo	0.2	10.48	23.49
2. S.Savory	0.2	26.08	15.83
3. Knowland	0.2	-1.29	-7.33
III. AntiRo			
1. C.Frayne	0.2	94.11	98.0
2. N.Gillard	0.2	-3.80	-2.18
3. D.Clement	0.2	19.66	10.46
4. M.Miller	0.2	18.64	15.03
5. Huggill	0.2	-0.76	4.69
6. E.Ashman	0.2	8.20	-4.52
7. T.Charles	0.2	-5.69	-11.56
8. Ravenhill	0.2	30.86	30.93
9. N.Cuff	0.2	13.84	29.22
IV. AntiSm/Ro			
1. M.Pryce	0.2	8.47	-17.18
V. AntiRo/La			
1. S.Davis	0.2	-2.44	2.17
2. M.Jone	0.2	15.66	23.54
3. S.Jone	0.2	3.48	17.12
4. P.Burnett	0.2	36.59	36.88
5. M.Rydor	0.2	-8.67	-6.50
VI. AntiRNP			
1. E.Swindell	0.2	1.22	20.65
2. M.Windsor	0.2	-2.29	6.74
3. R.Carter	0.2	-0.54	-3.92
4. Johanna	0.2	1.45	-2.36
5. A.Key	0.2	7.23	-11.79
6. M.Fry	0.2	-17.12	-8.38
7. E.Burness	0.2	-15.11	-20.17
VII. AntiSm/RNP			
1. P.Summes	0.2	21.32	20.93
VII. AntiSm			
1. P.Higginson	0.2	5.09	8.20
2. V.Norton	0.2	17.84	28.44
3. R.Hodges	0.2	-1.19	-11.11
IX. AntiDNA			
1. M.Holman	0.2	20.47	11.06
2. P.Plummer	0.2	6.03	8.06
3. J.Hildea A	0.2	8.91	19.88
4. J.Hildea B	0.2	-44.66	-24.79
5. F.Jonas	0.2	91.18	95.75

Note: The negative values of % inhibition represent stimulation of incorporation of radioactivity.

test), showed less inhibitory effect on TMV RNA translation than bound fraction. Therefore, the effect of ANAs on TMV RNA translation is still unclear.

5.2.3. Effect of ANAs on translation of various RNA templates in rabbit reticulocyte lysate cell-free systems.

5.2.3.a. The effect of ANAs on translation of globin mRNA.

38 ANA samples from SLE patients and 10 samples of IgG from normal individuals were used in this experiment. Some of these ANAs inhibited globin mRNA translation in reticulocyte lysate as shown by determination of the incorporation of radioactivity after incubation for 30 and 60 min in the presence of ANAs. For normal IgG samples the inhibition was compared to reaction mixtures without IgG as in the experiment with TMV RNA. The effect of normal IgG (10 samples) gave the mean value of inhibition of $-0.22 \pm 14.39\%$ and $-3.77 \pm 13.14\%$ at 30 min and 60 min incubation, respectively.

For ANA samples, it appeared that inhibition of globin synthesis did not relate to any particular type of antibodies; in the case of antiRo-and antiDNA-antibodies, only one sample in each group gave very high inhibition (95% at concentration of 2.2µg in 11µl reaction mixture). Most ANA samples showed similar inhibition to their effects on TMV RNA translation, except that some ANA samples gave slightly higher or lower percentage inhibition. All of these results are summarized and shown in Table 5.4 and Fig.5.8.

For gel anlysis, both gradient 5-15% SDS-PAG and 10% SDS-PAG were used to separate translation products and fluorograms are shown in Fig.5.9.a, b, c, and d. The major product was globin protein at M.W. of about 13K and some higher M.W. proteins (about 65K, 48K, 28K, and 21K) were also synthesized. The protein pattern on 5-15% SDS-PAGE showed more protein bands than 10% SDS-PAGE, including 68K

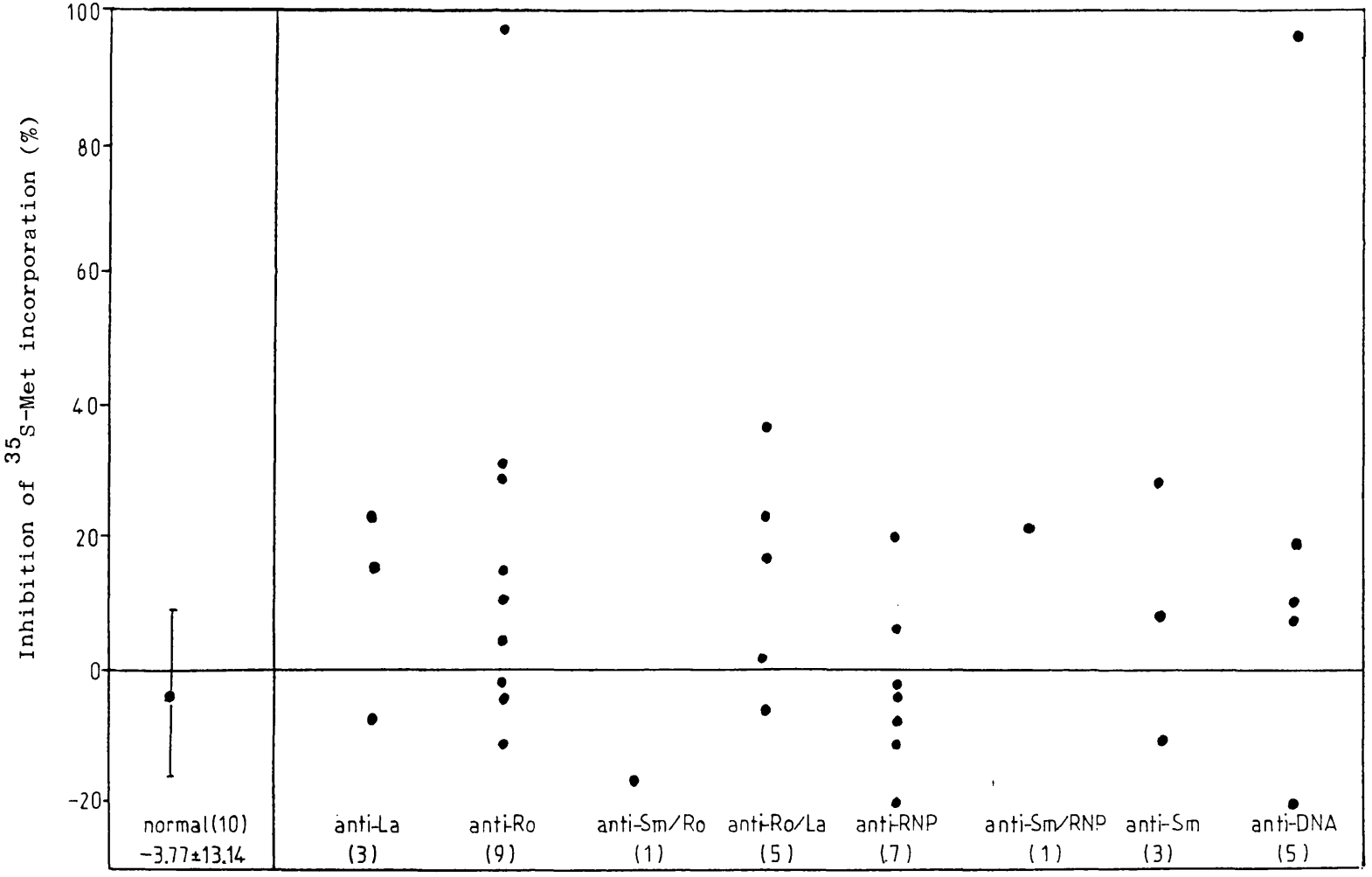


Fig. 5.8. Comparison of inhibition by ANAs from different SLE subgroups in translation of globin mRNA in rabbit reticulocyte lysate cell-free system. The mean value with standard deviation for normal IgG (10 samples) is shown as ⊤•⊥. All inhibition values for both ANAs and normal IgG were at 60 min of incubation.

Fig. 5.9. Analysis of the translation products of globin mRNA in reticulocyte lysate with IgG from normal and SLE patients by SDS-PAGE and followed by autoradiography (a) and fluorography (b-d). a and b were 5-15% SDS-PAGE while c and d were 10% SDS-PAGE

(a)
lane 1: control without mRNA
" 2: added H_2O
" 3: " normal IgG(W.M);0 2µg/µl
" 4: " " " (W.M);0.1 "
" 5: " antiLa(Buffalo);0 2 "
" 6: " " (Buffalo);0.1 "
" 7: " antiRo(Frayne);0.1 "
" 8: " " (Frayne);0.05 "
" 9: " antiRo(Davis);0.2 "

Exposure: 67,000-260,000 cpm;
20 days; -70°C.

(b)
lane 1: added normal IgG(NG)
" 2: " normal IgG(M.N)
" 3: " antiRo(Frayne)
" 4: " antiRo(Ravenhill)
" 5: " antiRo(Cuff)
" 6: " antiDNA(Jonas)
" 7: " antiDNA(Holman)
" 8: " antiSm(Norton)
" 9: " antiSm/RNP(Summes)
" 10: " antiRNP(Swindell)
" 11: " antiRo/La(M.Jone)
" 12: " standard proteins ^{14}C-BSA, ^{14}C-T.I, ^{14}C-RNase

Exposure: 12,000-250,000 cpm;
6 days; -70°C

(c)
lane 1: added normal IgG(K.T)
" 2: normal IgG(K.O)
" 3: " antiRo(Frayne)
" 4: " antiRo(Cuff)
" 5: " antiRo(Ravenhill)
" 6: " antiDNA(HildeaA)
" 7: " antiDNA(Plummer)
" 8: " antiRNP(Swindell)
" 9: " antiRNP(Windsor)
" 10: " antiRo/La(S.Jone)
" 11: " antiRo/La(M.Jone)
" 12: " standard proteins ^{14}C-BSA, ^{14}C-T.I ^{14}C-RNase

Exposure 4,000-96,000 cpm;
4 days; -70°C

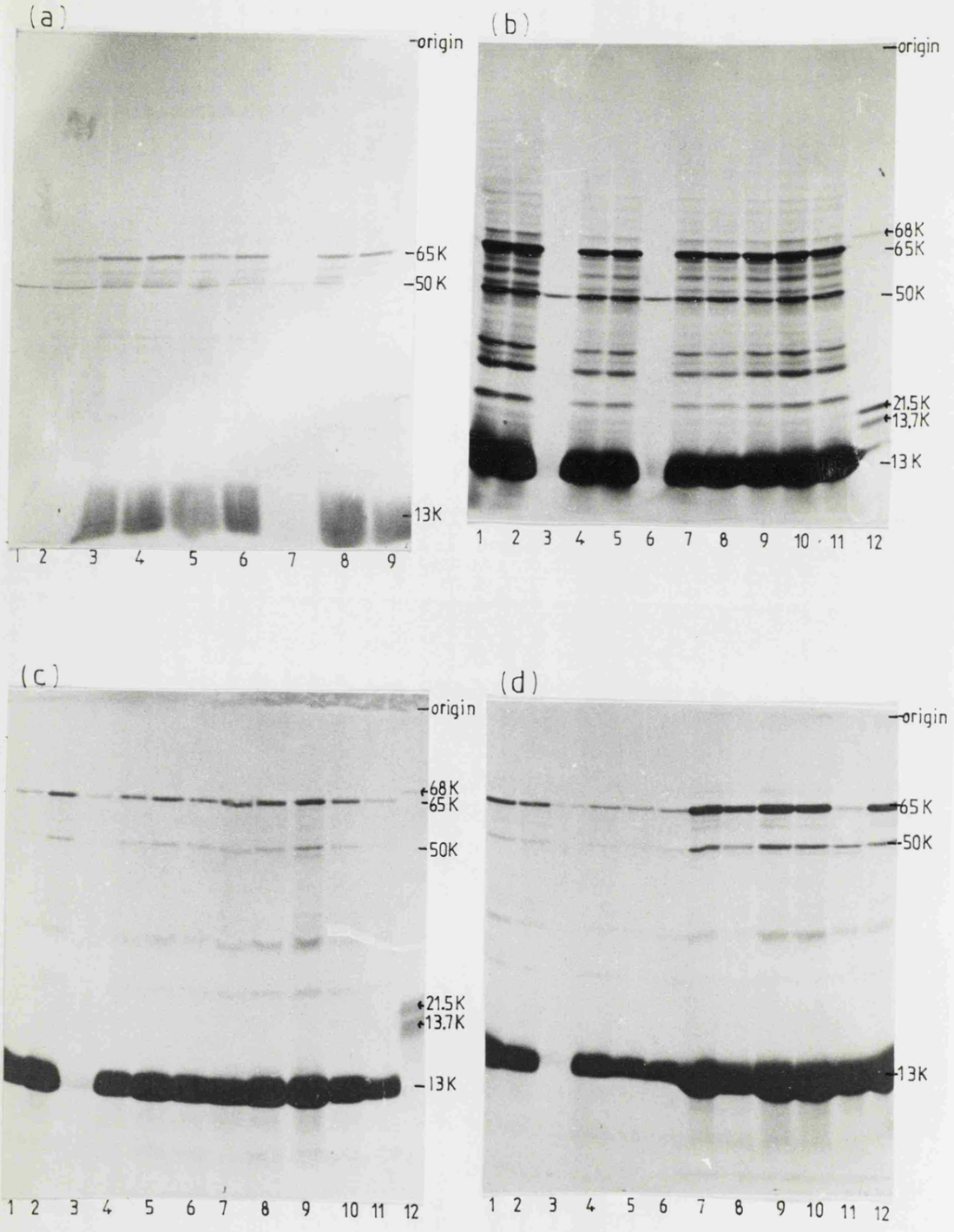
(d)
lane 1: added normal IgG(NG)
" 2: " normal IgG(M.N)
" 3: " antiDNA(Jonas)
" 4: " antiDNA(Holman)
" 5: " antiSm(Higginson)
" 6: " antiSm(Norton)
" 7: " normal IgG(Miss)
" 8: " antiRo(Clement)
" 9: " antiRo(Miller)
" 10: " antiSm/Ro(Pryce)
" 11: " antiRo/La(Davis)
" 12: " antiLa(Knowland)

Exposure: 1,700-43,000 cpm;
5 days; -70°C.

Note: In (b),(c), and (d), normal IgG and ANAswere added to reaction mixture at a concentration of 0.2 µg/µl.

T.I. = trypsin inhibitor

Fig. 5.9


(a)
origin
65K
50K
13K
1 2 3 4 5 6 7 8 9
(b)
origin
68K
65K
50K
21.5K
13.7K
13K
1 2 3 4 5 6 7 8 9 10 11 12
(c)
origin
68K
65K
50K
21.5K
13.7K
13K
1 2 3 4 5 6 7 8 9 10 11 12
(d)
origin
65K
50K
13K
1 2 3 4 5 6 7 8 9 10 11 12

60K, 55K, and 35K which may result from higher radioactivity of loaded samples (Fig. 5.9.a, b, and 5.9. c , d, respectively). There was no protein synthesis (except protein at M.W. about 47-48K which was also seen in a reaction mixture without mRNA, Fig. 5.9.a. lane 1) at high percentage inhibition (Fig. 5.9.a. lane 7, Fig. 5.9.b. lane 3 and 6, Fig.5.9.c. lane 3 and Fig.5.9.d. lane 3). At lower percentage inhibition, globin and other proteins were synthesized in lower amount when compared to control with added normal IgG but the result from the fluorogram of globin band did not show the difference which might be related to high amount of protein synthesized (Fig 5.9.a lane 5, Fig 5.9.b. lane 4, 5, and 8, Fig.5.9.c. lane 4,6, 11 and Fig. 5.9.d. lane 5, 6, 8).

5.2.3.b. The effect of ANAs on translation of poly(A)$^+$RNA from K562 and NS 1 cells.

68% (V/V) lysate was used to translate K562 poly(A)$^+$RNA in the presence of ANAs (antiRo (Frayne) and antiLa (Buffalo))at concentrations of 0.55 -2.2 µg in 11µl reaction mixture. Both types of ANA showed inhibition of incorporation of radioactivity into protein. The inhibition was dependent on the concentration of ANAs. For antiRo (Frayne), the inhibition was 10.35% and 38.9% at concentrations of 0.55µg and 1.1µg in 11µl reaction mixture, respectively. The same result was given by adding antiLa (Buffalo) at concentrations of 1.1 and 2.2µg in 11µl reaction mixture; inhibition of 25.4% and 32.7% was obtained.

For NS 1 poly(A)$^+$RNA, 60% (V/V) lysate was used. The inhibition was 43% for antiRo (Frayne) at a concentration of 1.1µg in 11µl reaction mixture and was only 16.8% for antiLa (Buffalo) at a concentration of 2.2µg in 11µl reaction mixture.

Table 5.5. Inhibition by ANAs of translation of poly u inarabbit reticulocyte lysate cell-free system.

Specificity / Name	Amount of IgG (μg/μl)	% Inhibition at 30 min	% Inhibition at 60 min
I. Normal			
1. W.Mullen	0.2	-1.54	6.32
2. K.Ounarom	0.2	-5.29	0.85
3. W.Y.NG	0.2	6.53	5.27
4. M.El-Naggar	0.2	-4.72	-4.89
5. K.Thompson	0.2	-16.83	-11.29
		-4.37±7.52	-0.75±6.59
II. AntiLa			
1. Buffalo	0.2	6.86±1.89	6.58±0.12
2. S.Savory	0.2	-17.13	-13.40
III. AntiRo			
1. C.Frayne	0.2	73.24±4.89	65.77±2.85
2. N.Gillard	0.2	8.43±3.25	3.24±10.32
3. Ravenhill	0.2	8.92	0.72
4. N.Cuff	0.2	-0.01±5.85	3.03±5.39
IV. AntiRo/La			
1. M.Jone	0.2	-6.36±1.7	-12.36±3.39
2. S.Jone	0.2	1.26±3.36	11.16±5.26
V. AntiRNP			
1. E.Swindell	0.2	-2.18±9.62	-5.21±3.08
2. M.Windsor	0.2	3.10	4.02
VI. AntiSm/RNP			
1. P.Summes	0.2	-1.81±2.09	4.02±5.76
VII. AntiSm			
1. P.Higginson	0.2	21.46±5.87	17.98±3.55
2. V.Norton	0.2	9.06±1.48	6.80±2.49
VII. AntiDNA			
1. M.Holman	0.2.	-19.11±6.8	-14.96±2.13
2. P.Plummer	0.2	8.37	9.46
3. J.Hildea A	0.2	25.63±6.78	16.54±0.23
4. F.Jonas	0.2	67.53±1.09	55.22±3.37

Note: The negative values of % inhibition represent stimulation of incorporation of radioactivity.

5.2.3.c. Translation of poly u affected by ANAs.

The ANAs which showed the inhibitory effect on translation of TMV RNA and globin mRNA, were used to investigate their effect on poly u translation. The inhibition by ANAs was calculated and is shown in Table 5.5. Some of the ANA samples showed a reduced level of inhibition while others showed stimulation. Poly u directed ^{3}H-Phe incorporation in the reticulocyte lysate system was extremely sensitive to protein synthesis inhibitors (both cycloheximide and aurin tricarboxylic acid), being 85% inhibited at 20μM.

These results showed that ANAsdid not inhibit translation of any specific types of RNA. For example, antiRo (Frayne) and antiLa (Buffalo) showed inhibition of incorporation of radioactivity regardless of the type of RNA whether TMV RNA, globin mRNA, poly(A)$^{+}$ RNA from K562 and NS 1 cells or poly u .

5.2.4. Effect of PBS concentration on translation of globin mRNA in rabbit reticulocyte lysate and inhibitory mechanism of ANAs.

5.2.4.a. Effect of PBS concentration on globin mRNA translation.

The rabbit reticulocyte lysate translation system for globin mRNA was sensitive to salt concentration. There was a low effect or no inhibition when using PBS at a concentration of 38μM and the inhibition increased with increasing salt concentration. The inhibition of incorporation of radioactivity was 32.34%, 68.36% and 91.59% when adding PBS at concentrations of 84, 210 and 420 μM, respectively, to the reaction mixture.

The same result was obtained as for TMV RNA translation

in the reticulocyte lysate system that the inhibition by ANAs was not due to changes in salt concentration. This was shown by comparing the conductivity of IgG solutions (ANA samples) to the inhibition obtained (Fig. 5. 10. a.). These ANAs and normal IgG were diluted with PBS and the inhibition increased at a high concentration of ANAs, while normal IgG showed no inhibitory effect at all concentrations. AntiDNA (Jonas), antiRo (Frayne), antiLa (Buffalo) and antiSm (Higginson) were used and the results are shown in Fig.5.10.a. The translation product from this experiment was analyzed on gel electrophoresis and the fluorogram is shown in Fig.5.10.b. In ANA samples which gave a very high percentage inhibition (70%) protein at M.W. 48K was synthesized together with quite a low amount of globin protein (M.W. 13K) (Fig. 5. 10. b. lane 3, 4, 9, and 10). Higher amounts of globin protein and protein at M.W. of 65K, 48K and 28K were synthesized in translation products at lower percentage inhibition (about 35%) (Fig 5 .10 . b. lane 5, 6, 11, 13 and 14).

5.2.4.b. Inhibition of globin protein synthesis by ANAs

Globin protein synthesis was studied in a time-course experiment. ANA(antiLa (Buffalo); antiRo (Frayne); antiDNA (Jonas)), PBS, normal IgG and protein synthesis inhibitors(cycloheximide and aurin tricarboxylic acid) were added to the reaction mixture after 10 min of incubation to give final concentrations of 0.2µg/µl and 50µM, respectively. The incubation was continued at 30°C and samples of the reaction mixture (1µl) were taken out at 15, 20, 30 and 60 min for radioactivity determination. The results showed that antiRo and antiDNA curves mimicked cycloheximide while antiLa gave a curve which mimicked aurin tricarboxylic acid (Fig. 5. 11. a.). The translation products were further analyzed on SDS-PAGE and the fluorogram is

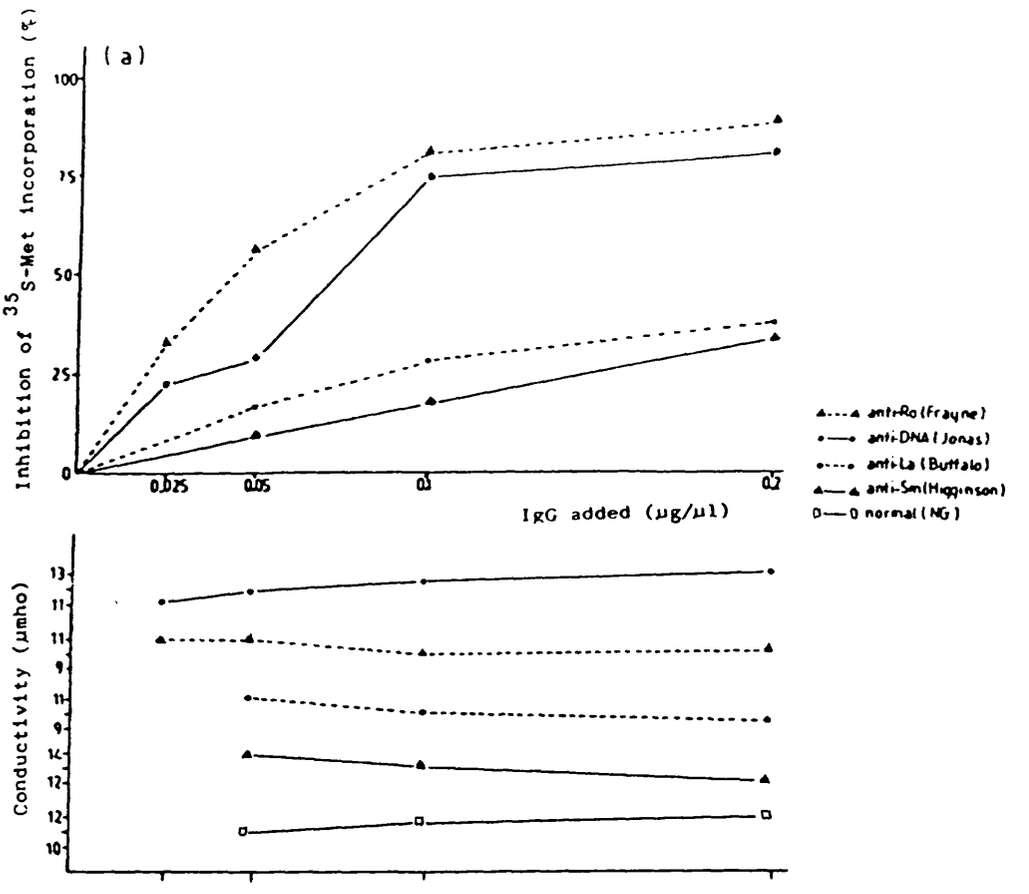


Fig. 5.10. Effect of ANA concentration on globin synthesis in the rabbit reticulocyte lysate cell-free system.

a) Comparison of percent inhibition at different concentration of ANAs with conductivity.

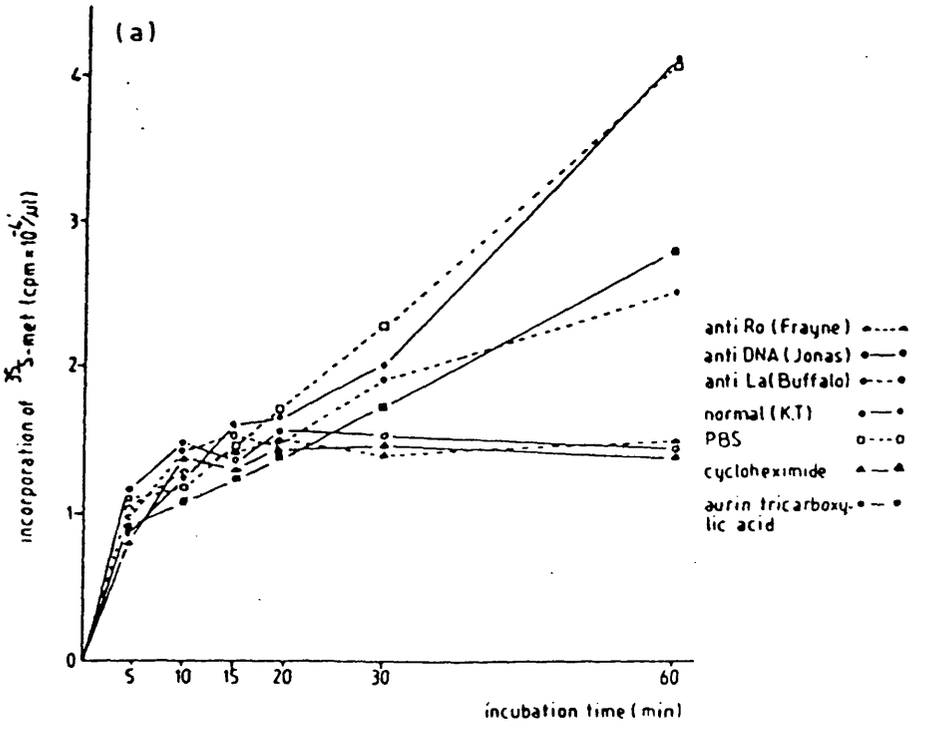


Fig. 5.11. Comparison of effectson globin synthesis in reticulocyte lysate by ANAs and protein synthesis inhibitors (cycloheximide and aurin tricarboxylic acid).

a) Time-course experiment, ANAs and protein synthesis inhibitors were added to reaction mixture at 10 min incubation.

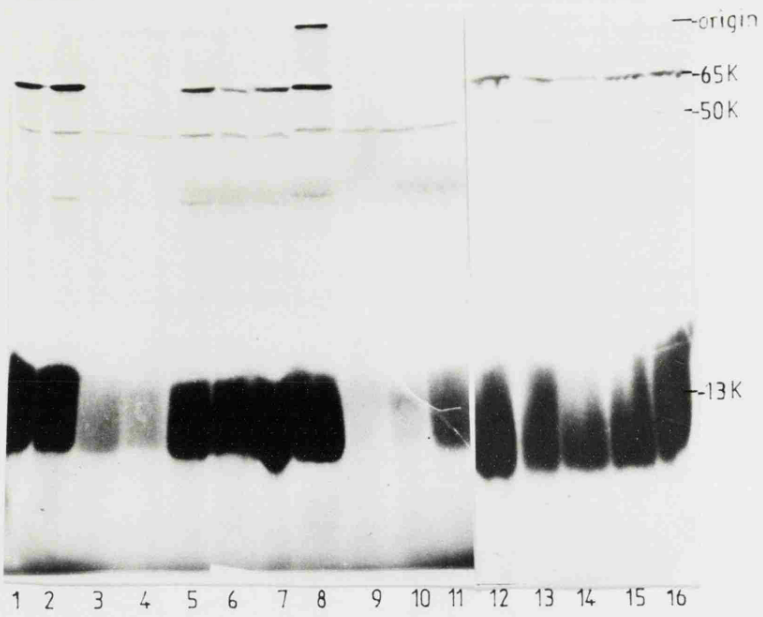


Fig. 5.10. b) Fluorogram of translation product from (a).

lane 1: added normal IgG(NG),0.2µg/µl lane 9: added antiDNA(Jonas),0.2µg/µl
" 2: " " " (NG),0.1 " 10: " " (Jonas),0.1 "
" 3: " antiRo(Frayne),0.2 " " 11: " " (Jonas),0.05 "
" 4: " " (Frayne),0.1 " " 12: " normal IgG(NG),0.05 "
" 5: " " (Frayne),0.05 " " 13: " antiSm(Higginson),0.2"
" 6: " antiLa(Buffalo),0.2 " " 14: " " (Higginson),0.1"
" 7: " " (Buffalo),0.1 " " 15: " " (Higginson),0.05"
" 8: " " (Buffalo),0.05" " 16: " antiRo(Frayne),0.025"

Exposure: 14,000-83,000 cpm; 1-2 days; -70°C.

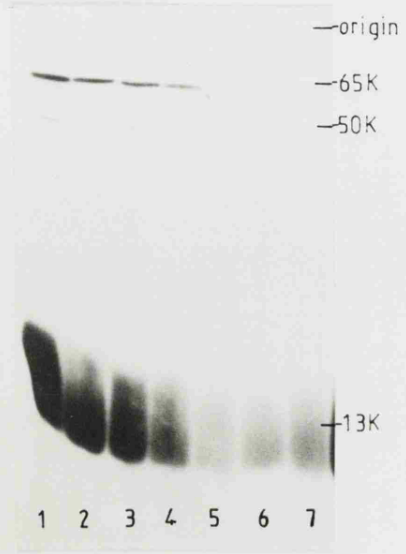


Fig. 5.11. b) Fluorographic pattern of translation product from (a).

lane 1: added PBS
" 2: " normal IgG(K.T)
" 3: " antiLa (Buffalo)
" 4: " aurin tricarboxylic acid
" 5: " cycloheximide
" 6: " antiRo (Frayne)
" 7: " antiDNA (Jonas)

Exposure: 29,000-83,000 cpm; 1 day; -70°C.

Table 5.6. Effect of ANAs on TMV protein synthesis in wheat germ lysate.

Specificity / Name	% Inhibition by ANAs at concn. of 0.2µg/µl		
	at 30 min	at 60 min	at 90 min
I. Normal			
1. W.Mullen	-7.7	-9.2	-6.4
2. K.Ounarom	13.85	-7.2	-3.15
3. W.Y.NG	8.59	-4.66	-14.98
4. M.El-Naggar	8.5	13.1	3.6
5. K.Thompson	1.3	-0.3	-7.4
6. Mc.Keowan	14.8	2.9	11.7
7. S.F.Chai	8.1	10.1	7.7
8 Mrs.M.	-0.89	-1.2	15.8
9. Miss	-14.16	-10.06	-27.97
10. B.Al-Ibrahim	-6.5	-25.6	0.54
	2.59±9.77	-3.21±11.03	-2.06±13.03
II. AntiLa			
1. Buffalo	24.20±20.6	49.05±3.7	74.35±17.89
2. S.Savory	-7.8	-3.1	-9.5
3. Knowland	25.5	28.9	30.8
III. AntiRo			
1. C.Frayne	66.70±15.84	73.60±5.66	90.40±1.27
2. N.Grillard	5.1	16.4	20.1
3. D.Clement	31.2	40.0	47.8
4. M.Miller	23.4	-3.2	14.4
5. Huggill	-0.41	25.9	15.9
6. E.Ashman	16.7	14.4	21.8
7. T.Charles	-5.4	-7.8	-26.8
8. Ravenhill	47.5	59.5	71.5
9. N.Cuff	32.7	48.2	43.2
IV. AntiSm/Ro			
1. M.Pryce	38.4	49.8	54.9
V. AntiRo/La			
1. S.Davis	3.7	6.4	46.9
2. M.Jone	9.1	30.5	39.7
3. S.Jone	8.2	23,2	39.8
4. P.Burnett	-8.3	-17.6	31.6
5. M.Rydor	19.6	11.5	9.1
VI. AntiRNP			
1. E.Swindell	-14.9	-10.3	-2.5
2. M.Windsor	37.8	43.1	53.9
3. R.Carter	46.3	61.8	70.6
4. Johanna	37.8	33.2	42.3
5. A.Key	-9.3	6.7	33.5
6. M.Fry	-1.6	14.9	24.1
7. E.Burness	0.97	-0.73	15.0
VII. AntiSm/RNP			
1. P.Summes	29.7	40.5	41.5
VIII. AntiSm			
1. P.Higginson	28.0	43.2	51.4
2. V.Norton	25.9	48.7	50.6
3. R.Hodges	0.87	17.8	24.9
IX. AntiDNA			
1. M.Holman	7.7	18.1	22.3
2. P.Plummer	-1.4	14.6	27.4
3. J.Hildea A	18.9	39.2	56.5
4. F.Jonas	51.75±21.4	60.65±19.73	76.40±20.79

Note: The negative values of % inhibition represent stimulation of incorporation of radioactivity.

shown in Fig. 5.11.b.

The results did not clearly differentiate between inhibition at initiation and elongation steps of protein synthesis, unlike the results with TMV RNA. This may be due to the low M.W. of major synthesized product, globin protein (about 13K). In samples which showed inhibition at elongation step only the amount of globin synthesized was less and there was no protein synthesis at M.W. of 65K (Fig. 5.11. b. lane 5 -7). Smaller amounts of globin protein than control were also seen in samples which were inhibited at the initiation step (Fig. 5.11.b. lane 3 and 4).

5.2.5. Effect of ANAs on TMV RNA translation in wheat germ lysate.

5.2.5.a. Inhibitory effect of ANA on TMV RNA translation.

The optimum conditions for translation of TMV RNA were described in section 4.1.1.c. In this experiment, TMV RNA was translated in wheat germ lysate in the presence of ANAs or normal IgG at a concentration of 0.2µg/µl and incorporation of radioactivity was determined at 30, 60, and 90 min of incubation. All ANA samples and normal IgG were used as experiments on TMV RNA translation with rabbit reticulocyte lysate.

The inhibition index was calculated by comparing ANAs to normal IgG while normal IgG was compared to reaction mixture without any added IgG. These results are summarized in Table 5.6 and Fig.5.12. The normal IgG sample showed no effect on TMV RNA translation and the mean values of percent inhibition were 2.59 ± 9.77, -3.21 ± 11.03 and -2.06 ± 13.03 at 30, 60 and 90 min of incubation, respectively. Most ANA samples gave higher inhibition of TMV protein synthesis than in reticulocyte lysate. For example, antiRNP groups showed inhibition of 15-70% while in reticulocyte lysate the percent inhibition

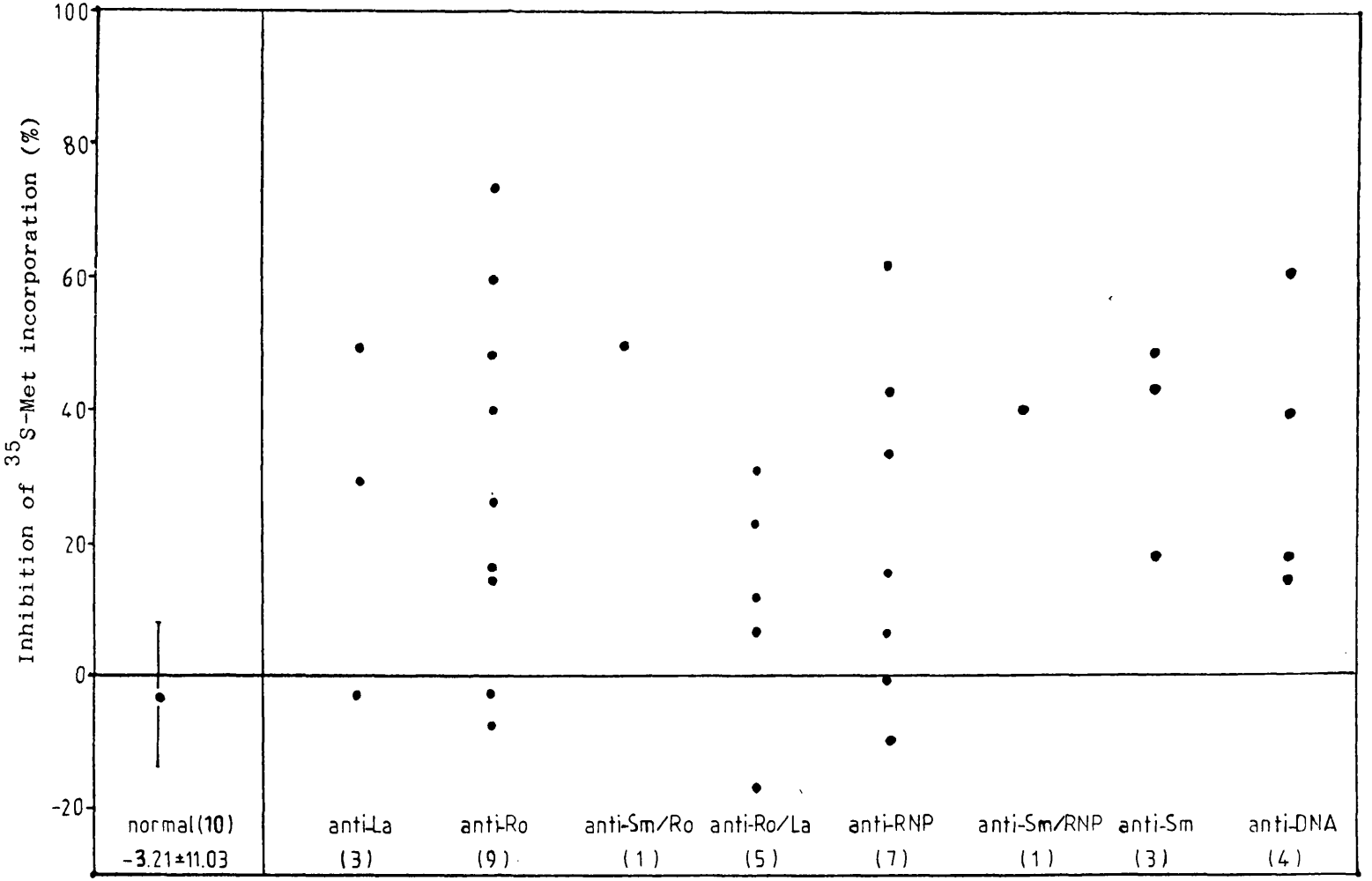
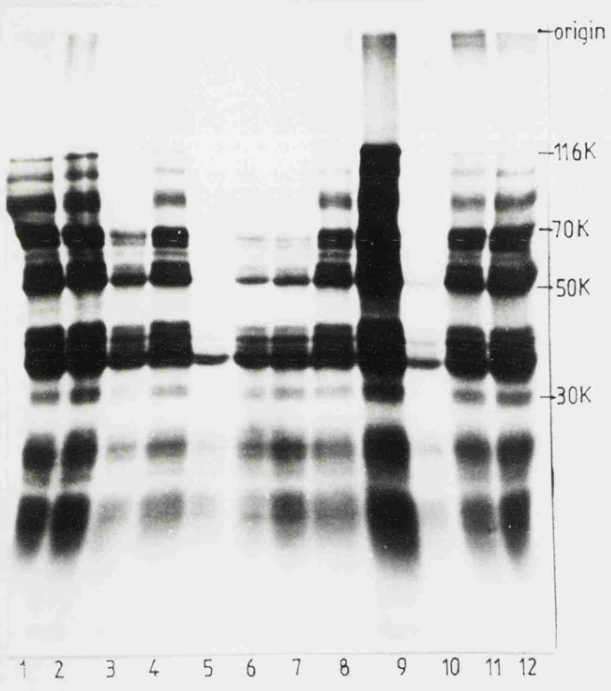


Fig. 5.12. Comparison of inhibition by ANAs from different SLE subgroups on translation of TMV RNA in wheat germ lysate. The mean value with standard deviation is shown for normal IgG samples (10)($\overset{\mid}{\bullet}$). All inhibition values for both ANAs and normal IgG were at 60 min of incubation.

Fig. 5.13. A fluorograph of SDS-PAGE of translation product from incubations in the presence of ANAs with ^{35}S-Met for 90 min, at 25^{o}C.

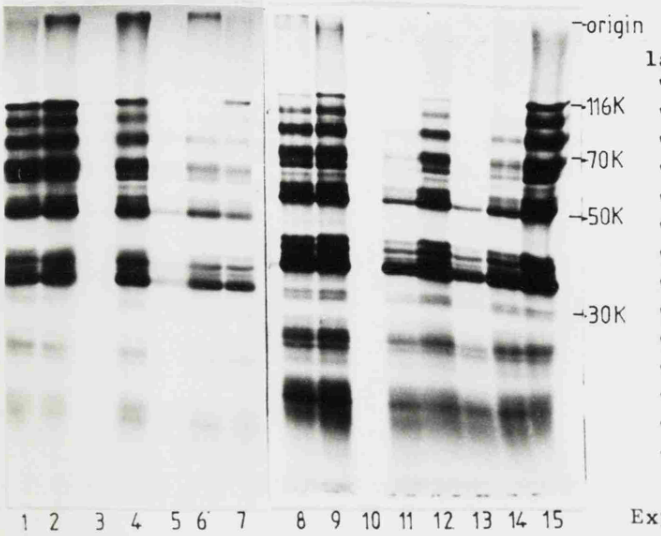
(a)



lane 1: added normal IgG(B.I)
" 2: " antiRNP(Swindell)
" 3: " " " (Windsor)
" 4: " " " (Fry)
" 5: " " " (Carter)
" 6: " " " (Johanna)
" 7: " antiSm/Ro(Pryce)
" 8: " antiRo (Ashman)
" 9: " PBS
" 10:: " antiSm(Higginson) at concn. of 0.2µg/µl
" 11: added antiSm(Higginson) at concn. of 0.1µg/µl
" 12: added antiSm(Higginson) at concn.of 0.05µg/µl

Exposure: 30,000-120,000 cpm; 12 days; -70^{o}C.

(b)



lane 1: added normal IgG(K.O)
" 2: " " " (K.T)
" 3: " antiLa(Buffalo)
" 4: " " " (Savory)
" 5: " " " (Knowland)
" 6: " antiDNA(Holman)
" 7: " " " (Plummer)
" 8: " normal IgG(NG)
" 9: " " " (Miss)
" 10: " antiRo(Frayne)
" 11: " " " (Clement)
" 12: " " " (Gillard)
" 13: " " " (Ravenhill)
" 14: " " " (Cuff)
" 15: " " " (Charles)

Exposure: 9,000-260,000 cpm; 5 and 10 days; -70^{o}C.

was only 5-8%. This may result from the efficiency of the wheat germ lysate system. The efficiency of wheat germ lysate for translation of TMV RNA was lower than the reticulocyte lysate system, as shown by the larger proportion of low M.W. protein product and the absence of protein bands at M.W. of 176K in gel analysis (Fig.5.13 a. lane 1, Fig.5.13.b. 1,2,8 and 9 and 5 and Fig 5.2.a. lane 2 and 3).

The inhibitory effect of ANAs on TMV RNA translation was confirmed by gel analysis. Fluorograms of SDS-PAGE of translation product in the presence of normal IgGs and ANAs are shown in Fig.5.13. a and b. The amount of protein synthesized was dependent on the percentage inhibition, at high percentage inhibition there was no protein synthesis (Fig. 5.13.b. lane 3 and 10) and synthesis of lower M.W. protein started at a lower percent of inhibition (Fig.5.13.a. lane 3, 6, 7, 10 and Fig.5.13 b. lane 11, 13, and 14).

5.2.5.b. Effect of PBS concentration on TMV RNA translation.

In the reticulocyte lysate cell-free system, high concentrations of PBS (K^+, Na^+, HPO_4^{2-} and Cl^- ions) can inhibit the translation of TMV RNA in the wheat germ lysate system, which is more sensitive to this effect. As shown in Fig 5.14., PBS at a concentration of about 10.5μM (or conductivity value of 3.8μmho) showed no effect on TMV RNA translation in the wheat germ lysate system, but the inhibition was increased to 7.5%, 29%, 76.8% and 81.1% when using PBS at concentrations of 7.2, 12.2, 27.5, and 52.0μM, respectively. The conductivity of ANA samples ranged from 2.5 to 9.0μmho. Therefore, the inhibitory effect on TMV RNA translation should not be related to salt concentration, except for some samples which had conductivity greater than 4μhmo, where the effect may result from either ANAs or salt concentration.

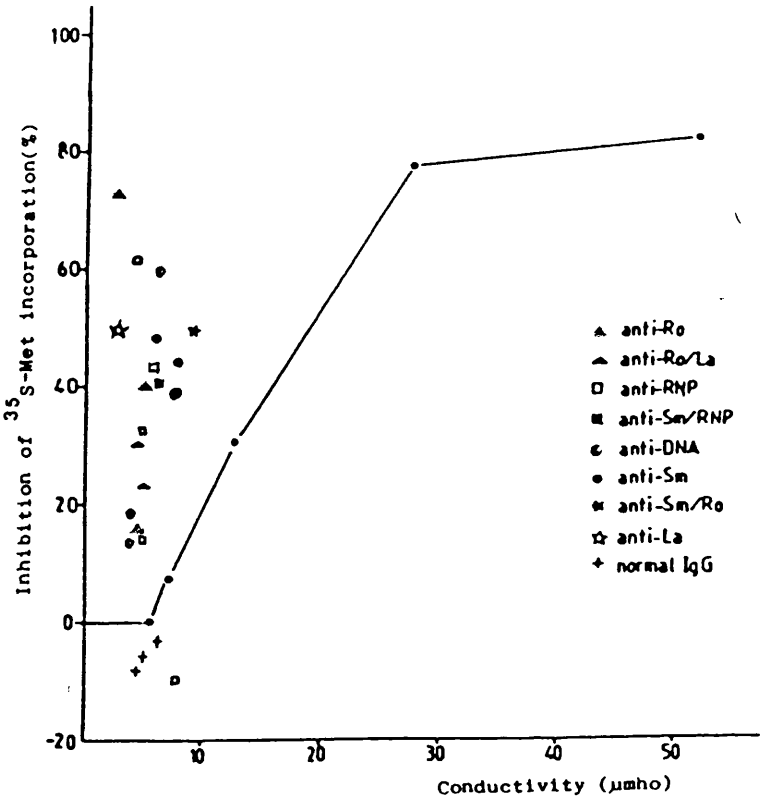


Fig. 5.14. Effect of PBS on translation of TMV RNA in wheat germ lysate: Comparison of percentage inhibition with conductivity of IgG solution. IgG from both normals and SLE patients(at concn. of0.2 μg/μl) and various concentrations of PBS were added to the reaction mixture and incorporation of radioactivity was determined at 60 min incubation. The inhibition was dependent on PBS concentration, shown as conductivity value(•—•). The conductivity of ANA samples was determined and related to their percentage inhibitions.

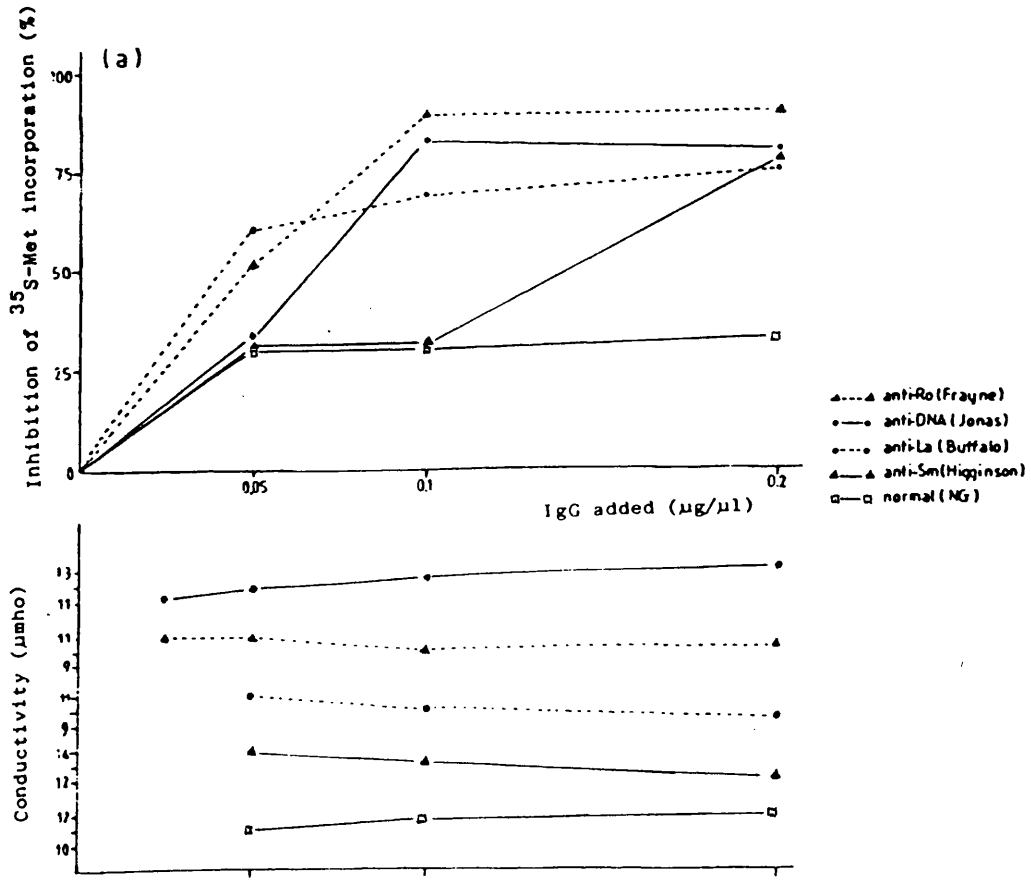
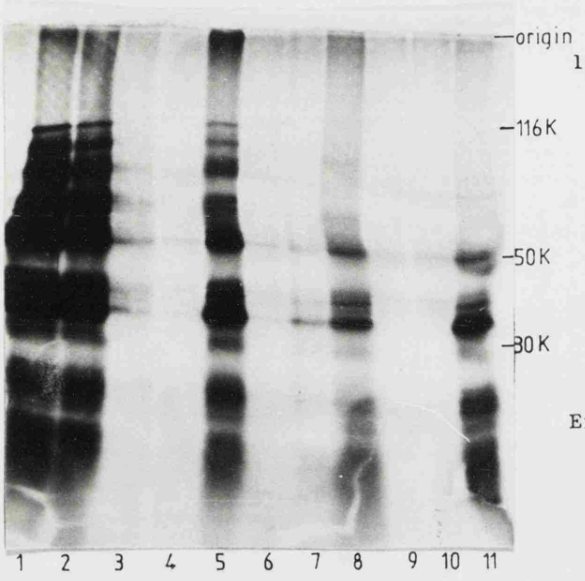


Fig. 5.15. TMV RNA directed protein synthesis in wheat germ lysate in the presence of ANAS and normal IgG at different concentration.

a) Percentage inhibition and conductivity compared to the concentration of ANAS in reaction mixture.

Fig. 5.15. b and c) Fluorogram of labelled TMV protein synthesized in the presence of ANAs, in wheat germ lysate (b and c) and reticulocyte lysate (c).

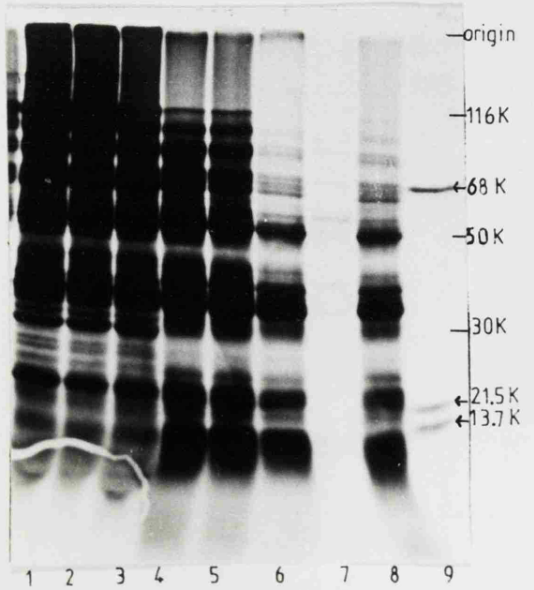
(b)



lane 1: added normal IgG(NG);0.2µg/µl
" 2: " " " (NG):0.1 "
" 3: " antiRo(Frayne);0.2 "
" 4: " " (Frayne);0.1 "
" 5: " " (Frayne);0.05 "
" 6 " antiLa(Buffalo);0.2 "
" 7: " " (Buffalo):0.1 "
" 8: " " (Buffalo);0.05"
" 9: " antiDNA(Jonas);0.2 "
" 10: " " (Jonas);0.1 "
" 11: " " (Jonas);0.05 "

Exposure: 15,000-100,000 cpm;
20 days; -70°C.

(c)



lane 1-3 (in reticulocyte lysate)
" 4-8 (in wheat germ lysate)
" 1: added normal IgG(K.T)
" 2: " " " (NG)
" 3: " antiLa(Buffalo).
" 4: " H_2O
" 5: " PBS at concn.of 84µM
" 6: " PBS at " " 210µM
" 7: " PBS at " " 420µM
" 8: " normal IgG(NG)
" 9: " standard proteins

Exposure: 24,000-200,000 cpm;
4 days; -70°C.

From experiments on translation of TMV RNA in the presence of ANAs and normal IgG diluted with PBS, it was clearly shown that the inhibition was dependent on the amount of added ANAs(Fig.5.15.a.). In this experiment, normal IgG at concentrations from 0.05 to 2.2µg/µl (conductivity ranging from 11.5 to 12.2µmho) gave nearly constant percentage inhibition, about 30%. This inhibition should not be related to the amount of IgG added. Because the wheat germ system was more sensitive to salt concentration, PBS at a concentration of 84µM (conductivity 12.2µmho) gave 29% inhibition. Therefore, the inhibition of TMV RNA translation by normal IgG was due to salt concentration.

The translation products were further analyzed on SDS-PAGE and fluorographic patterns are illustrated in Fig. 5.15.b and c. The results demonstrated that synthesis of proteins of all sizes was inhibited by ANAs and high M.W. protein is seen to be affected prior to lower M.W. protein.

5.2.6. Detection of the antigenically active protein in cell lysates using immunoblotting technique.

The inhibitory effect of ANAs on protein synthesis in cell-free systems was shown with various types of RNA templates and in both wheat germ and reticulocyte lysate systems. Using specific antiRo and antiLa with TMV RNA translation in the rabbit reticulocyte lysate system, the relationship between inhibitory effect and antibody specificity was unclear. (in result section 5.2.2.f.).

The immunoblotting technique was shown to be efficient in detecting antigenically active proteins after separation by gel electrophoresis. Many laboratories have used this technique to identify the antigenic proteins detected by antiRo, La, Sm and RNP. The results are varied, which might be due to the source of antigens used or degradation and aggregation of antigens during their preparation (Steitz

Table 5.7. Antigenic proteins of Ro, La, Sm, and Sm/RNP recognized by their antibodies(ANAs)using the immunoblotting technique.

Specificity	Sample	M.W.of antigenic proteins(K) in crude antigen	M.W.of antigenic proteins(K) in purified antigen	reticulocyte lysate	wheat germ lysate
AntiLa	Buffalo(sera)	45,31	52,48,46,(35),32	50.5	N.D
"	Buffalo(IgG)	N.D	48,45.5,43,30	49.0	N D
"	Knowland(sera)	57	69,49.5,46.5,45,30	(64),(62),52	N.D
AntiRo	Frayne(sera)	(61),53	63,(54)	negative	negative
"	Frayne(IgG)	(76),53,(26)	N.D	"	N.D
"	Cuff(sera)	(66),53,(48)	66,(62),52,(48)	"	negative
"	Clement(sera)	(76),63,(61),56,53,(26)	N.D	"	N.D
"	Gillard(sera)	53	N.D	"	negative
AntiRNP	Windsor(sera)	104,(86),(33.5)	97,(65.5),(62),(55),32,30.5	53.0	N.D
"	Swindell(sera)	(32),31.5,(30.5)	(64),(60),(34),31.5,30.5,(29)	59,31.5	N.D
"	Carter(sera)	32	33,31.5	negative	N.D
"	Fry(sera)	(32),31.5,(30.5)	N.D	32.0	N.D
AntiSm	Hamilton(sera)	(230,122,83,(58),(48)	117.5,(64),(58)	N.D	N.D
"	Morrison(sera)	N.D	66,62	N.D	N.D

Note: The M.W of antigenic proteins was acculately determined and presented as the mean value of duplicate estimations. The M.W in a bracket was a faint band. Calf thymus extract was used as crude antigen for antiLa, antiSm, and antiRNP. Human spleen extract was used as crude antigen for antiRo.
N.D=not determined

et al.,1982; hite et al.,1982; MacGillivray et al.,1982; Wooley et al., 1983).

In this experiment, antigenic proteins in either crude or purified antigen preparations and both reticulocyte lysate and wheat germ lysate were identified using ANAs which showed inhibitory effects on protein synthesis in the cell-free translation systems. The proteins from crude or purified antigen and reticulocyte or wheat germ lysate was separated on 5-15% SDS-PAGE prior to transfer to nitrocellulose sheets. The antigenic proteins were detected by double antibody technique. Specific antibodies were used, followed by peroxidase-conjugated anti-human IgG (as described in detail in section 5.1.5).

AntiLa (Buffalo and Knowland), antiRo (Frayne, Clement, Cuff and Gillard), antiRNP (Windsor, Swindell, Carter and Fry) and antiSm (Hamilton and Morrison) were used together with calf thymus extract, human spleen extract and the purified antigens Ro, La, Sm and RNP/Sm (provided by Dr. P. Maddison, Royal National Hospital for Rheumatic Diseases, Bath and Mr. D. M. Brennand in this department).

The antigenic proteins recognized by these ANA samples are summarized in Table 5.7. Both antisera and IgG fractions from SLE patients were used and were shown to react with the same proteins (Fig.5.16.a. lane 1 and 3 and Fig.5.16.b. lane 3, 4, and 5). Different protein antigens are recognized by antisera from different SLE patients. For example, antiSm (Hamilton) reacts with protein at M.W. of 117K, 64K and 58K while antiSm (Morrison) recognized proteins at M.W. of 66K and 62K (Fig.5.16.c. lane 1 and 2). Crude antigen and purified antigen also gave slightly different result. AntiLa recognized many more antigenic proteins in purified antigen than in calf thymus extract (Fig.5.16.a. lane 5, 6, 8 and 9) and antiRNP also gave

Fig. 5.16. Immunoreactive proteins in crude tissue extract, purified antigen and cell-free translation lysate Proteins from these sourecs were separated on 5-15% SDS-PAGE and then transferred electrophoretically to nitrocellulose sheets. Each antigen was incubated with its appropriate antibody (antisera or IgG fraction from SLE patient) and then detected by using peroxidase-conjugated antihuman IgG. All antisera were used at dilution of 1/250,and 1/1,000dilution was used for peroxidase-conjugated antihuman IgG.

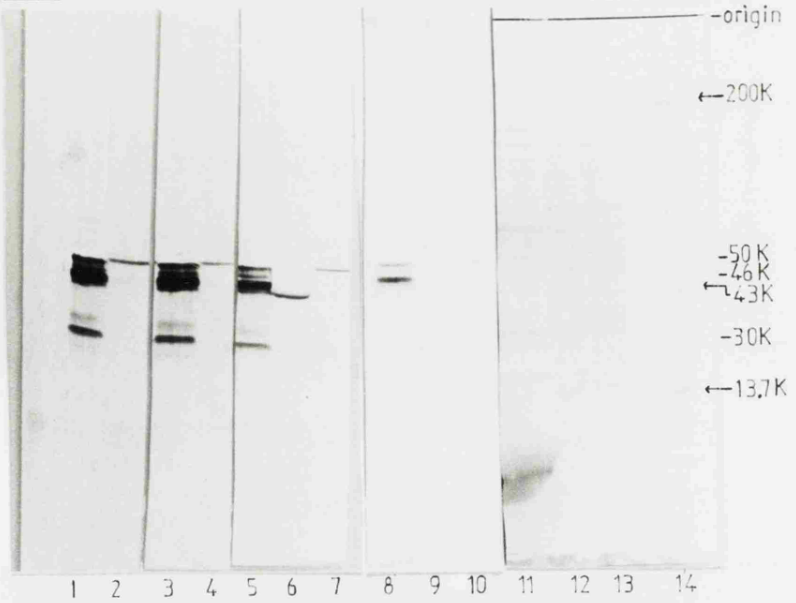
a) lane 1: antigen La reacted with antisera La (Buffalo)
" 2: reticulocyte lysate reacted with antisera La(Buffalo)
" 3: antigen La reacted with antiLa(Buffalo),IgG fraction
" 4: reticulocyte lysate reacted with antiLa(Buffalo),IgG fraction
" 5: antigen La reacted with antisera La(Buffalo)
" 6: calf thymus extract reacted with antisera La(Buffalo)
" 7: reticulocyte lysate " " " " "
" 8: antigen La reacted with antisera La(Knowland)
" 9: calf thymus extract reacted with antisera La(Knowland)
" 10: reticulocyte lysate " " " " "
" 11-14: protein transferred to nitrocellulose and stained with amido black.
" 11: reticulocyte lysate
" 12: calf thymus extract
" 13: calf thymus extract (30-60% $(NH_4)_2SO_4$ cut)
" 14: standard proteins: myosin(200K); ovalbumin(43K); trypsin inhibitor(21.5K); and RNase(13.7K).

b) lane 1: wheat germ lysate reacted with antisera Ro(Frayne)
" 2: reticulocyte lysate " " " " "
" 3: antigen Ro reacted with antisera Ro(Frayne)
" 4: human spleen extract reacted with antisera Ro(Frayne)
" 5: human spleen extract reacted with antiRo(Frayne),IgG fraction
" 6: reticulocyte lysate " " " " " "
" 7: antigen Ro reacted with antisera Ro(Cuff)
" 8: human spleen extract reacted with antisera Ro(Cuff)
" 9: reticulocyte lysate " " " " "
" 10: human spleen extract reacted with antisera Ro(Clement)
" 11: reticulocyte lysate " " " " "
" 12: standard proteins: myosin(200K): BSA(68K); ovalbumin(43K); trypsin inhibitor(21.5K); and RNase(13.7K).

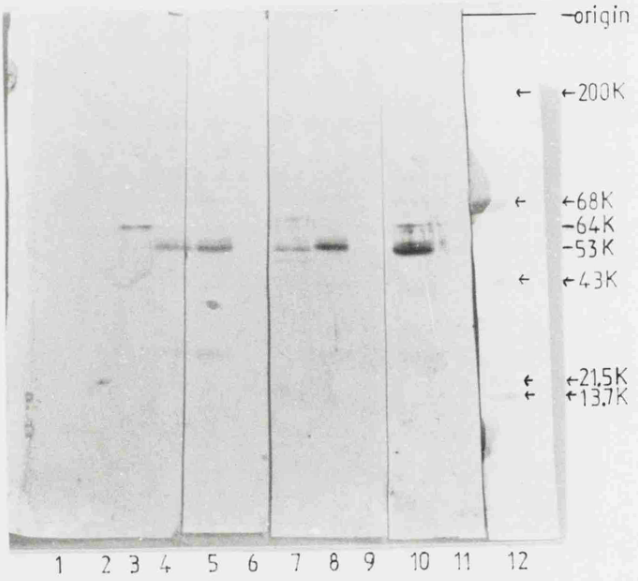
c) lane 1: antigen Sm reacted with antisera Sm(Hamilton)
" 2: " " " " antisera Sm(Morrison)
" 3: calf thymus extract reacted with antisera RNP(Windsor)
" 4: antigen RNP/Sm reacted with antisera RNP(Windsor)
" 5: reticulocyte lysate reacted with antisera RNP(Windsor)
" 6: calf thymus extract " " antisera RNP(Swindell)
" 7: antigen RNP/Sm reacted with antisera RNP(Swindell)
" 8: reticulocyte lysate reacted with antisera RNP(Swindell)
" 9: calf thymus extract reacted with antisera RNP(Fry)
" 10: reticulocyte lysate " " " " "
" 11: calf thymus extract reacted with antisera RNP(Carter)
" 12: antigen RNP/Sm reacted with antisera RNP(Carter)
" 13: reticulocyte lysate reacted with antisera RNP(Carter)
" 14: standard proteins; myosin(200K); BSA(68K); ovalbumin(43K); trypsin inhibitor(21.5K); and RNase(13.7K).

Fig.5.16

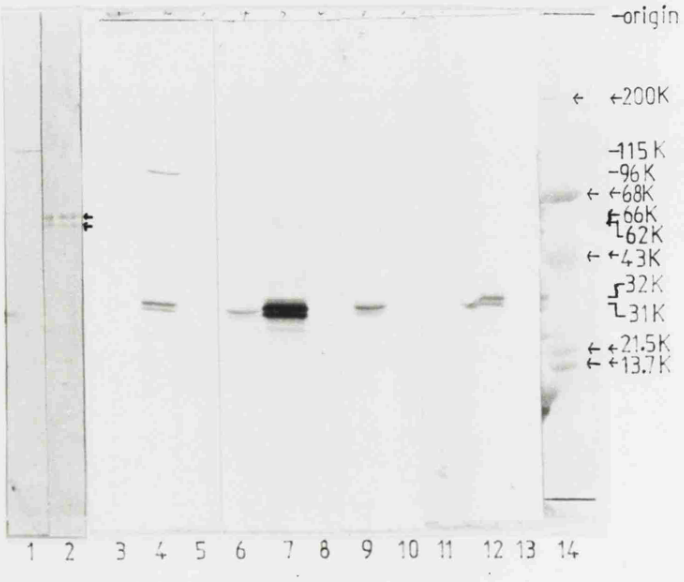
(a)


origin
200K
50K
46K
43K
30K
13.7K
1 2 3 4 5 6 7 8 9 10 11 12 13 14

(b)


origin
200K
68K
64K
53K
43K
21.5K
13.7K
1 2 3 4 5 6 7 8 9 10 11 12

(c)


origin
200K
115K
96K
68K
66K
62K
43K
32K
31K
21.5K
13.7K
1 2 3 4 5 6 7 8 9 10 11 12 13 14

the same result as antiLa (Fig.5.16.c. lane 3, 4, 6 and 7). The additional bands in purified antigen preparations might well result from degradation during the purification process.

All antiLa samples reacted with antigenic protein with M.W. of approximately 50K in reticulocyte lysate (Fig.5.16.a. lane 2,4,7 and 10). AntiRo samples did not recognize any protein in either wheat germ or reticulocyte lysate. Moreover, with antiRNP samples, 3 out of 4 samples gave a reaction with proteins of M.W. 59K, 53K and 32K (Fig. 5.16.c. lane 5, 8 and 10) in reticulocyte lysate. The details of these results are shown in Table 5.7. The lack any bands in lysate might be related to a low amount of antigenic protein which cannot be detected by this technique.

Chapter Six Effect of ANAs on protein synthesis in intact cells.

6.1. Methods

6.1.1. Radiolabelling of protein in cell lines with ^{35}S-Met and extraction of labelled proteins.

6.1.1.a. Pulse-labelling of protein in cells

6.1.1.b. Extraction of labelled protein from cells.

6.1.1.c. Determination of radioactivity.

6.1.2. Transfer of IgG molecules into intact cells.

6.1.2.a. Using Liposomes.

-Preparation of liposomes.

-Incubation of cells with liposomes.

6.1.2.b. Using red cell ghosts.

-Preparation of red cell ghosts by pre-swell technique.

-Microinjection of IgG molecules into cells.

6.1.2.c. Via Fcγreceptors.

6.1.2.d. By permeabilization.

6.1.3. Testing the efficiency of transfer of IgG molecules into cells using fluorescein-labelled IgG(FITC-IgG) and ^{125}I-labelled IgG.

-Using FITC-IgG.

-using ^{125}I-labelled IgG.

6.2. Results

6.2.1. Labelling of proteins in cells by ^{35}S-Met.

6.2.2. Effect of ANAs on protein synthesis in intact cells.

6.2.2.a. Using liposomes to transfer ANAs into cells.

i)Effect of liposomes on viability of cells and incorporation of ^{35}S-Met by cells.

ii)Efficiency of liposomes to entrap and transfer macromolecules into cells.

-using cytochrome c.

-using ^{125}I-labelled IgG.

-using FITC-IgG.

iii)Effect of ANAs on protein synthesis in intact cells.

iv)Effect of cycloheximide on protein synthesis in K562 cells.

6.2.2.b. Using red cell ghosts to introduce ANAs into cells.

6.2.2.c. Antibody penetration of cells through Fcγ receptors.

6.2.2.d. Introduction of ANAs into cells by permeabilization in the presence of ANAs.

i) Effect of permeabilization on the viability and incorporation of radioactivity into cells.

ii) Efficiency of K562 cells in take up of macromolecules by permeabilization procedure.

-Using ^{125}I-labelled IgG.

-Using FITC-IgG.

-Effect of ANA on protein synthesis.

6. Effect of ANAs on Protein Synthesis in Intact Cells.

6.1. Methods.

In these experiments, some of the ANA samples which showed inhibitory effects on protein synthesis in cell-free systems, including antiRNP-antibodies, were used to examine their effects on intact cells. Several techniques were used to transfer IgG molecules into intact cells, including fusion with liposomes or red cell ghosts, transfer via Fcγ receptors on cells and permeabilization of cells. The details of this method will be discussed in this chapter together with radiolabelling of cellular protein and extraction and determination of radioactivity of labelled proteins. These radiolabelled proteins were further analyzed on SDS-PAGE either single-dimensional or two-dimensional gels. Gel electrophoresis techniques and fluorography were described in detail in section 2.2.7.

6.1.1. Radiolabelling of Protein in Cell Lines with ^{35}S-Met and Extraction of Labelled Proteins.

The methods described by Mishell and Shiigi(1980) and Johnston and Thorpe (1982) were used with some modifications.

6.1.1.a. Pulse-labelling of protein in cells.

About 1-2.5 X 10^6 cells were washed twice with met-free medium. The cells were collected by centrifugation at 300g for 10 min, resuspended in 1ml of met-free medium which contained 2mM glutamine and 10% FCS, and incubated with 5-10μCi of ^{35}S-Met at 37°C in a 5% CO_2 in air-mixture, for 3 to 48hr.

In time-course experiments, 10μl of samples (triplicate) were taken out at 1, 2, 3 and 4 hr to determine radioactivity.

After completion of the incubation, the cells were harvested and washed twice with 2-5ml of ice-cold washing buffer (0.01M phosphate buffer saline pH7.2 containing 0.02% NaN_3 and 2mg/ml of met)

to remove the excess free ^{35}S-Met. The last wash was transferred to 12 x 75 mm plastic tubes and duplicate 10µl samples were withdrawn to determine radioactivity of whole cells. The cells were collected and further used to extract protein. Acid-insoluble radioactivity in the medium was also determined and the rest was kept at -70°C for gel analysis.

6.1.1.b. Extraction of labelled protein from cells.

Washed cells in 12 x 75mm plastic tube, completely free of supernatant, were mixed with 0.05-0.2ml of extraction buffer (10mM Tris-HCl pH7.2 containing 0.15M NaCl, 0.02% NaN_3 and 0.5% (W/V) of NP40) on a vortex mixer and left on ice for 15 min. The mixture was transferred to a plastic microfuge tube and centrifuged at high speed in MSE microcentrifuge, for 10-15 min. The supernatant (extracted protein) was collected and duplicate 2µl samples were used for radioactivity determination. This extracted protein was kept at -70°C for further analysis by gel electrophoresis. The recovery of extracted protein from cells was calculated.

6.1.1.c. Determination of radioactivity.

The sample (2-50µl) was mixed with 0.5ml of 0.9% NaCl containing 0.5% FCS. The protein was precipitated by adding 0.5ml of 10% TCA containing 2mg/ml of met. The mixture was left at room temperature, for at least 30 min before filtering on a GF/C filter. The protein precipitate was washed with 10ml of 5% TCA and 5ml of ethanol, dried and radioactivity counted in 3ml of scintillation fluid.

6.1.2. Transfer of IgG Molecules into Intact Cells.

6.1.2.a. Using liposomes.

This was performed according to the method of Lenk et al. (1982). Some modifications were used in the preparation of liposomes.

Preparation of liposomes.

0.32ml of phosphatidyl choline (10mg/ml in chloroform) was mixed with 0.02ml of cholesterol (10mg/ml in chloroform) and diluted to 1ml with ether. The aqueous solution (about 0.2ml PBS, ANAsor normal IgG) was slowly added into the organic solvent while mixing on a vortex mixer, and mixing was continued for 2-5 min. The solution was then shaken in a sonicator bath during the evaporation of organic phase with nitrogen gas. The organic phase was completely separated out by suction with a water pump for 5-10 min. The liposome pellet was resuspended in 1ml of PBS and collected by centrifugation at 5,000 rpm for 10 min. These liposomes were washed twice with PBS and resuspended in 1ml PBS before use.

Incubation of cells with liposomes.

About 2-4 X 10^6 cells were harvested, washed twice with PBS, resuspended in 1ml of liposomes in PBS and incubated at room temperature for 30 min. For 2 hr incubation, this cell suspension was diluted with 1ml of culture medium and incubation continued at 37°C in a 5% CO_2 in air-mixture while for 18 hr incubation, the cells were diluted to 400,000 cells/ml with culture medium. The viability of cells was determined as described in section 2.2.1.b. These cells were harvested and protein labelled with ^{35}S-Met as described in section 6.1.1.a.

6.1.2.b. Using red cell ghosts.

Preparation of red cell ghosts by pre-swell technique.

This method was performed according to Schlegel and Rechsteiner (1978). Human red blood cells were separated from 2ml of heparinized blood and washed three times with 5ml of ice-cold PBS (Dulbecco's formula without Ca^{2+} and Mg^{2+}). White blood cells and residual protein were carefully removed during the washing. The washed red blood cells were resuspended in PBS (Dulbecco's formula without Ca^{2+} and Mg^{2+}) at the concentration of 50% (V/V). 0.3ml of this cell suspension was added into 11ml of 55% (V/V) of PBS (Dulbecco's formula without Ca^{2+} and Mg^{2+}) to swell the cells and the pellets were collected by centrifugation at 300g, for 10 min. About 0.2ml of this pellet was mixed with 0.1ml of PBS or IgG solution from normal and SLE patients followed by addition of 0.2ml of sterile water to lyse the cells. After standing on ice for 2-3 min, 30µl of 10fold concentrated PBS (Dulbecco's formula without Ca^{2+} and Mg^{2+}) were added and the mixture was incubated at 37°C, for 60 min. These loaded red cells (or red cell ghosts) were washed 3 times with PBS by centrifugation at 600g, for 10 min.

Microinjection of IgG molecules into cells.

About 4×10^6 of washed cells were mixed with red cell ghosts and 1ml of 55% (W/W) polyethylene glycol 4000 (in PBS containing 5% (V/V) of DMSO) was slowly added while gently mixing the suspension. PBS was slowly added to the mixture until the final volume was 25-30ml. The cell pellets were collected by centrifugation at 300g for 10 min.and washed twice with PBS. These washed cells were resuspended in 1ml of culture medium and incubated at 37°C with a 5% CO_2 in air-mixture, for 2 hr. The

red cell ghosts were fused to cells by the procedure of Boogaard and Dixon (1983 a).

The viability of cells was determined as described in section 2.2.1.b. while the rest of these cells were used to label proteins with ^{35}S-Met as described in section 6.1.1.a.

Before extraction of labelled protein, the red cells were separated by mixing cell pellets with lysis buffer (10mM $KHCO_3$ containing 0.155M NH_4Cl and 0.1mM EDTA) at 90% (V/V). After standing at room temperature for 5 min, the mixture was resuspended in 4ml of PBS, overlayed on to 2ml of albumin solution (5% (W/V) in PBS) and centrifuged at 300g for 10 min. The cell pellets were collected and washed twice with PBS. This is similar to the method used by Johnston and Thorpe (1982).

6.1.2.c. via Fcγ receptors.

About 1 X 10^6 K562 cells (which have a high percentage of Fcγ receptors at the cell membrane), in 0.1ml of culture medium was incubated with 0.1ml of PBS or IgG solution from normal and SLE patients, at 37°C, for 1 hr. The cells were adjusted to a concentration of 2 X 10^6 cells/ml and 400,000 cells/ml and incubation continued at 37°C in a 5% CO_2 in air-mixture, for 2 hr and 19 hr, respectively. The cells were harvested and pulse-labelled with ^{35}S-Met (section 6.1.1.a.). The viability of cells was also determined (as in section 2.2.1.b.).

6.1.2.d. By permeabilization.

About 2-4 X 10^6 cells were washed twice with PBS (Dulbecco's formula without Mg^{2+} and Ca^{2+}) and resuspended in this PBS at concentration of 50% (V/V). 0.015ml of this cell suspension

was added to 0.5ml of 60% PBS (V/V) to swell the cells and centrifuged at 300g, for 10 min. The cell pellets were mixed with 0.01ml of PBS or IgG solution from normal and SLE patients, followed by addition of 0.02ml of sterile water. The mixture was left on ice for 2-3 min, immediately mixed with 3µl of 10 fold concentrated PBS (Dulbecco's formula without Mg^{2+} and Ca^{2+}) and incubated at 37°C, for 1 hr. 1ml of culture medium was added and incubation was continued at 37°C in 0.5% CO_2 in air-mixture for 3 hr. The cells were harvested, pulse-labelled with ^{35}S-Met and viability determined as described in section 6.1.1.a. and 2.2.1.b., respectively. This permeabilization technique was adapted from the method of Schlegel and Rechsteiner (1978).

6.1.3. Testing the Efficiency of Transfer of IgG Molecules into Cells Using Fluorescein-labelled IgG (FITC-IgG) and ^{125}I-labelled IgG.

Using FITC-IgG.

About 18mg of human IgG in PBS were incubated with 0.2ml of fluorescein isothiocyanate solution (FITC, at a concentration of 5mg/ml in carbonate-bicarbonate buffer pH 9.5), at room temperature, in dark, for 2 hr. Unreacted FITC was removed by gel filtration on G25-Sephadex (medium) in PBS. The bound FITC-IgG was pooled, concentrated by Millipore Minicon ultrafiltration unit and absorbance determined at 280 and 495nm. The concentration of FITC-conjugate was calculated from the equation :-

$$\text{FITC-protein (mg/ml)} = \frac{A_{280} - (0.36 \times A_{495})}{1.4}$$ (Goldman, 1968).

The F/P ratio was estimated from the nomogram given by Wells et al. (1966).

The FITC-IgG had a protein concentration of 8.4mg/ml and

Table 6.1. Labelling cells with ^{35}S-Met

Cell line	Viability(%)	Incubation time	^{35}S-Met incorporation (cpm/cell)	^{35}S-Met incorporation (cpm/µl medium)	recovery of extraction(%)
RPMI1788	93.5	4 hr	3.14	607	38.48
"	-	1 day	3.85	1,019	-
"	74.6	2 days	2.69	1,384	44.68
RPMI8226	92.6	4 hr	4.06	160	42.96
"	-	1 day	4.44	295	-
"	68.9	2 days	4.82	1,235	40.25
HMy2	91.8	4 hr	4.48	155	43.86
"	79.8	1 day	3.66	265	49.27
K562	95.9	3 hr	4.29	409	41.25

Note: % recovery of extraction was calculated from $\frac{\text{total cpm of extracted protein}}{\text{total cpm of cells}} \times 100$

the ratio of F/P was 3.7. This FITC-IgG was used to examine the efficiency of transfer of IgG molecules in to cells by liposomesand permeabilization.

Using ^{125}I-labelled IgG.

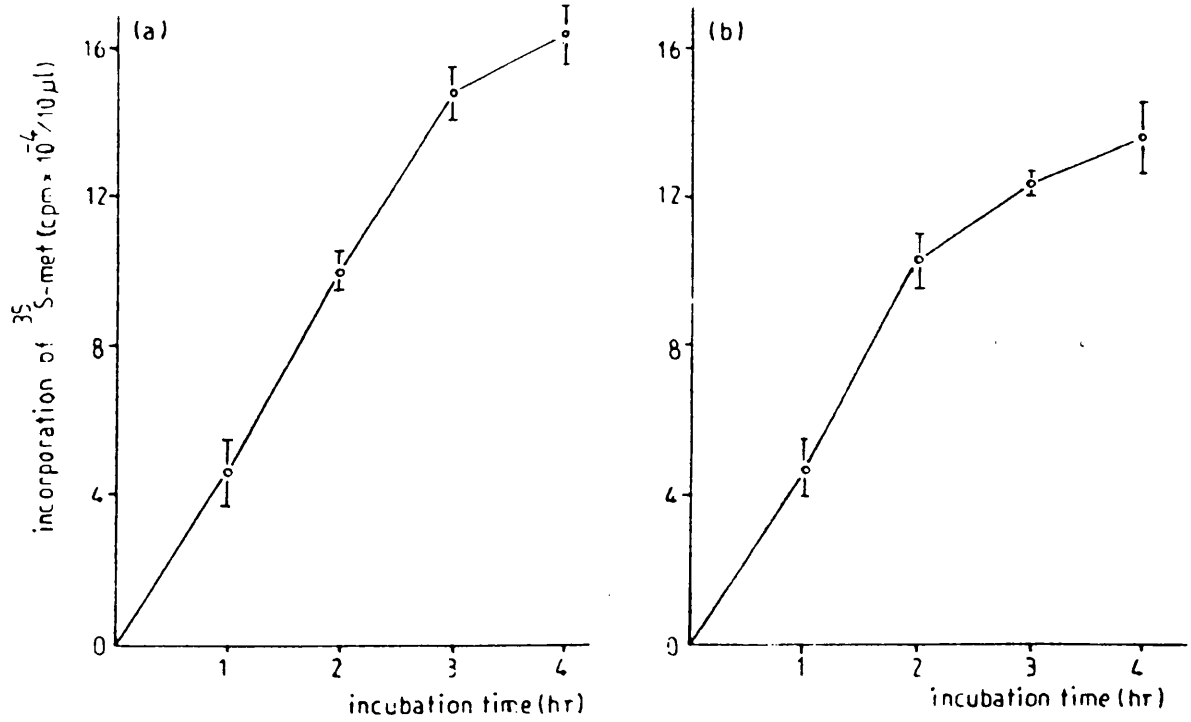
As with FITC-IgG, this ^{125}I-labelled sheep IgG was used to prepare liposomes or red cell ghosts and incubated with cells. The amount of ^{125}I-labelled sheep IgG transferred into cells was determined and used to compare the efficiency of each technique.

6.2. Results.

6.2.1. Labelling of Proteins in Cells by ^{35}S-Met.

The type of cell line and optimal conditions for labelling protein were examined in this experiment. Four human cell lines, RPMI 1788, RPMI 8226, HMy2 and K562 cells were tested. The washed cells, which had viability greater than 90%, were incubated in the presence of ^{35}S-Met as described in method section 6.1.1.a.Incorporation of ^{35}S-Met increased linearly until 3hr of incubation and then levelled off as shown in Fig.6.1.a,b,and c.The incorporation of ^{35}S-Met after 3 and 4 hr incubations was similar and gave enough radiolabelled protein for gel analysis. Moreover, after incubation of the cells (RMPI 1788, RMPI 8226 and HMy2) for 1-2 days, the viability was decreased which may result in detection of a false amount of secreted protein in medium (Table 6.1.). Therefore, 3 hr incubation of cells with ^{35}S-Met was chosen for further studying.

The incorporation of ^{35}S-Met into cells was dependent on the concentration of radioactivity (Fig 6.2.). For 1 X 10^6 cells, 5μCi of ^{35}S-Met per 1 X 10^6 cells were used which resulted in



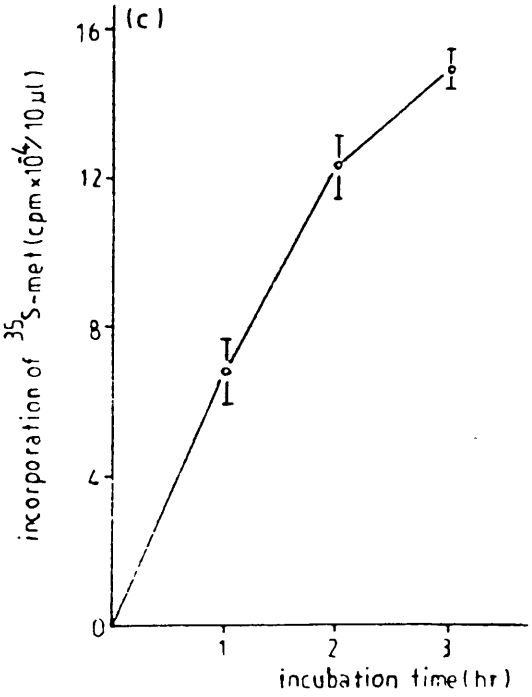


Fig. 6.1. Time-course of ^{35}S-Met incorporation by cell lines.

Cells in met - free medium were incubated with ^{35}S-Met at 37°C and triplicate 10 µl samples were taken to determine incorporation of radioactivity at 1hr intervals.

a) RPMI1788 cells(5×10^6) in 2 ml medium and 50µCi of ^{35}S-Met.

b) RPMI8226 cells(5.2×10^6) in 2 ml medium and 25µCi of ^{35}S-Met.

c) K562 cells(2.5×10^6) in 1 ml medium and 12.5µCi of ^{35}S-Met.

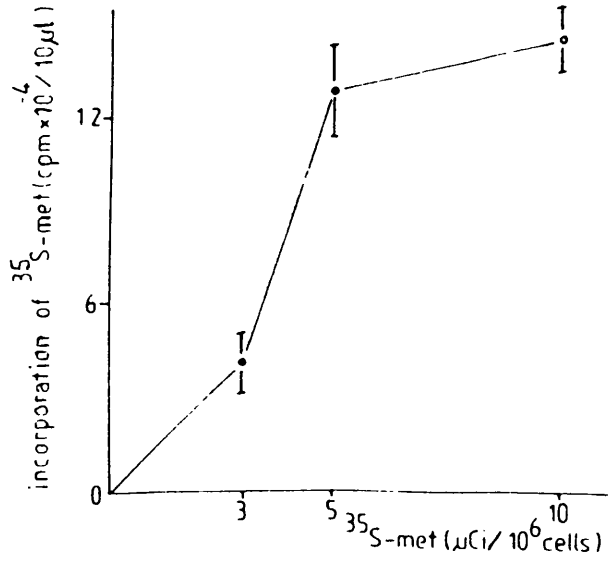


Fig. 6.2. Incorporation of ^{35}S-Met by RPMI1788 cells at various concentrations of ^{35}S-Met 10µl sample(25,000 cells) were taken after 4 hr incubation at 37°C and incorporation of radioactivity was determined.

incorporation of about 3-4cpm of ^{35}S-Met per cell after 4 hr incubation and the recovery of extracted protein from cells was between 38% and 44% (Table 6.1).

Single-dimensional gel electrophoretic analysis of labelled proteins from these cell lines followed by fluorography revealed a similarity of protein synthesis patterns(Fig. 6.3.a and b). The cell lines synthesized many proteins at M.W. range between 210K and 18K, including the intense band at M.W. of 42K which should be actin and they also showed their specificity of protein synthesis and secretion. For RPMI 1788 cells, which synthesized IgM, heavy chain (μ) at M.W. of 72K and light chain (λ) at M.W. of 25K were found in either cell lysate or medium (Fig.6.3.a. lane 1-5 and Fig.6.3.b. lane 1-4). Similar results were shown in RPMI 8226 cells, light chain (λ) was seen but the intensity of this band in medium was lower than in cell lysate (Fig.6.3.a. lane 6-9). Heavy chain (γ) at M.W. of 52K and light chain (k) at M.W. of 24K were synthesized and found in cell lysate from HMy2 cells (Fig.6.3.b. lane5-8) but the intensity of the bands was low. The extracted protein and whole cell lysate were loaded on to gels and it was found that the gel pattern from both samples was similar but the bands of extracted protein showed a sharper and clearer line (Fig 6.3.b. lane 1,3,5,7, and 9 for whole cell extract and lane 2, 4,6 , 8, and 10 for extracted protein). Therefore,protein extracted from cells was used for analysis on gel electrophoresis. Since the recovery of radioactivity in extracted protein varied slightly in each sample, equal amounts of radioactivity from each sample were used for gel analysis.

The extracted protein from cells and the precipitated protein from medium were directly separated on gel electrophoresis.

Fig. 6.3. Fluorogram of labelled proteins from cell lysate and culture medium after separation on 5-15% SDS-PAGE.

a) ^{35}S-Met labelled proteins from RPMI1788 and RPMI8226 cells. Labelled cells were lysed and protein precipitated from medium was dissolved in electrophoresis buffer, heated and analysed on 5-15% SDS-PAGE.

lane 1: RPMI1788 cells($5x10^6$ with 50µCi, ^{35}S-Met), 4 hr incubation
" 2: " " (" " 15µCi, "), 4 hr "
" 3: " medium(" " " , "), 4 hr "
" 4: " cells(" " 25µCi, "), 2days "
" 5: " medium (" " " , "), 2days "
" 6: RPMI8226 cells($5x10^6$ with 25µCi, ^{35}S-Met), 4 hr incubation
" 7: " medium (" " " , "), 4 hr "
" 8: " cells(" " " , "), 2 days "
" 9: " medium (" " " , "), 2 days "
" 10: standard proteins: ^{14}C-BSA, ^{14}C-trypsin inhibitor, ^{14}C-RNase, and reduced normal human IgG.

Exposure: 9,000-800,000 cpm; 1 day; -70°C.

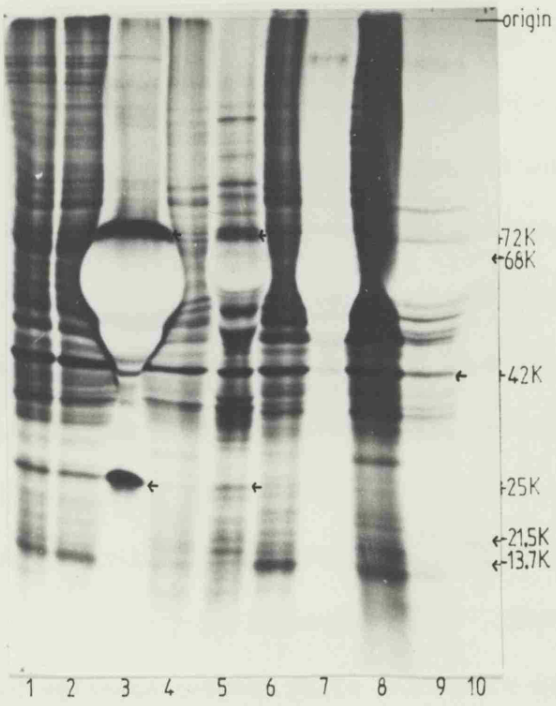
b) Comparison of radiolabelled protein from RPMI1788 and HMy2 cells incubated in medium with and without FCS: analysis of cell lysate and extracted protein on 5-15% SDS-PAGE.

lane 1: RPMI1788 cells($2.5x10^6$ with 12.5µCi, ^{35}S-Met), 4 hr incubation in medium with FCS.
" 2: RPMI1788 extracted protein($2.5x10^6$ with 12.5µCi, ^{35}S-Met) 4 hr incubation in medium with FCS.
" 3: RPMI1788 cells($2.5x10^6$ with 12.5µCi, ^{35}S-Met), 4 hr incubation in medium without FCS.
" 4: RPMI1788 extracted protein($2.5x10^6$ with 12.5µCi, ^{35}S-Met) 4 hr incubation in medium without FCS.
" 5: HMy2 cells($2.5x10^6$ with12.5µCi, ^{35}S-Met), 4 hr incubation in medium with FCS.
" 6: HMy2 extracted protein($2.5x10^6$ with 12.5µCi, ^{35}S-Met), 4 hr incubation in medium with FCS.
" 7: HMy2 cells($2.5x10^6$ with 12.5µCi, ^{35}S-Met), 4 hr incubation in medium without FCS
" 8: HMy2 extracted protein($2.5x10^6$ with 12.5µCi, ^{35}S-Met), 4 hr incubation in medium without FCS.
" 9: K562 cells($1x10^6$ with 5µCi, ^{35}S-Met), 3 hr incubation in medium without FCS.
" 10: K562 extracted protein($1x10^6$ with 5µCi, ^{35}S-Met), 3 hr incubation in medium without FCS.
11: standard proteins: ^{14}C-BSA, ^{14}C-trypsin inhibitor, ^{14}C-RNase

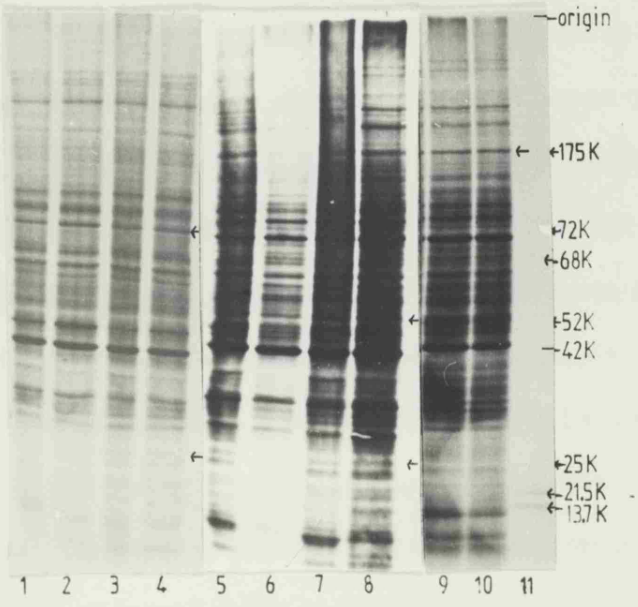
Exposure: 136,000-195,000 cpm; 20 hr - 3 days; -70°C.

Fig.6.3.

(a)


origin
72K
68K
42K
25K
21.5K
13.7K
1 2 3 4 5 6 7 8 9 10

(b)


origin
175K
72K
68K
52K
42K
25K
21.5K
13.7K
1 2 3 4 5 6 7 8 9 10 11

Because the amount of protein in medium with FCS was high, a distorted gel pattern was seen when the secreted protein in medium was analyzed (Fig. 6.3.a. lane 3 and 5). For this reason medium without FCS was used and the effect of this medium on radiolabelling of protein in HMy2 and RPMI 1788 cells was studied. There was little difference in incorporation of radioactivity into cellular protein when incubated in medium with FCS and without FCS. For HMy2 cells, 4.91 cpm of ^{35}S-Met were incorporated per cell after 3 hr incubation period in medium with FCS and this value decreased to 4.44 when cells were incubated in medium without FCS. The result was similar for RPMI 1788 cells, 4.41 cpm and 4.08 cpm of ^{35}S- Met were incorporated per cell at 3 hr incubation of cells in medium with FCS and without FCS, respectively. These labelled proteins were characterized on gel electrophoresis. There was no difference in the gel patterns between cells which were incubated in medium with FCS and without FCS (Fig. 6.3.b.lane 1,2,5,and 6 and lane 3,4,7,and 8 , respectively).

The result that RPMI 1788 and HMy2 cells synthesized IgM_{λ} and IgG_{k}, respectively, was confirmed by immuno-precipitation analysis. Rabbit antihuman-$IgM_{k,\lambda}$ and rabbit antihuman-IgG_{k} were used as antibodies to react with the labelled IgM and IgG from either cell lysates or medium and staphylococcus aureus (SaC), protein A-antibody adsorbent was added for rapid isolation of immunoprecipitate from the mixture as described by Mishell and Shiigi (1980)(slightly modified by adding normal serum to remove non-specific binding protein to SaC before adding antiserum). The yield of labelled IgM that was bound to SaC was 1.29 ± 0.31% (n=3) of total labelled protein in extracted protein from cells and this value was 0.04 ±0.01% (n=3) of total labelled protein in medium.

The same result was achieved for HMy2 cells, about 1.29 ± 0.31% (n=3) of total labelled protein was the labelled IgG in extracted protein from cells which bound to SaC and only 0.03 ± 0.01% (n=3) of total labelled protein was precipitated from the medium. The major heavy chains(μ or γ) and light chain (λ) were found together with low amounts of other proteins on gel analysis (data not shown). These results showed that these cells (RPMI 1788, HMy2) synthesized IgM or IgG molecules and SaC could separate out the antigen-antibody complex but also had non-specific binding to other protein and the percentage recovery of immunoprecipitate was rather low.

From the above results of characterization of the labelled protein from cell lineson single-dimensional gel electrophoresis, RPMI 1788 cells (both cell lysate and medium) seemed to show a high amount of heavy chain (μ) and light chain (λ) on gel pattern (Fig.6.3.a. lane 1-5). Therefore,RPMI 1788 cells were chosen for further study of the effect of ANAs on protein synthesis in intact cells.

The high resolution technique of two-dimensional gel electrophoresis was also used to analyze the labelled proteins of cells, since similar protein patterns for these cell lines were shown on single-dimensional gel electrophoresis. The method of 2D gel anlysis of labelled protein was as described in section 2.2.7.b. The 2D gel technique gave a reliable result. Similar gel patterns were shown when using labelled proteins from cells which had been incubated for different periods (Fig.6.4.a for 4 hr and Fig.6.4.b. for 24 hr), in medium with and without FCS (Fig.6.4. c and d.), with different amounts of radioactivity ^{35}S-Met (Fig.6.4. c and e) and running electrophoresis at different occasions (Fig. 6.4.c.and f).

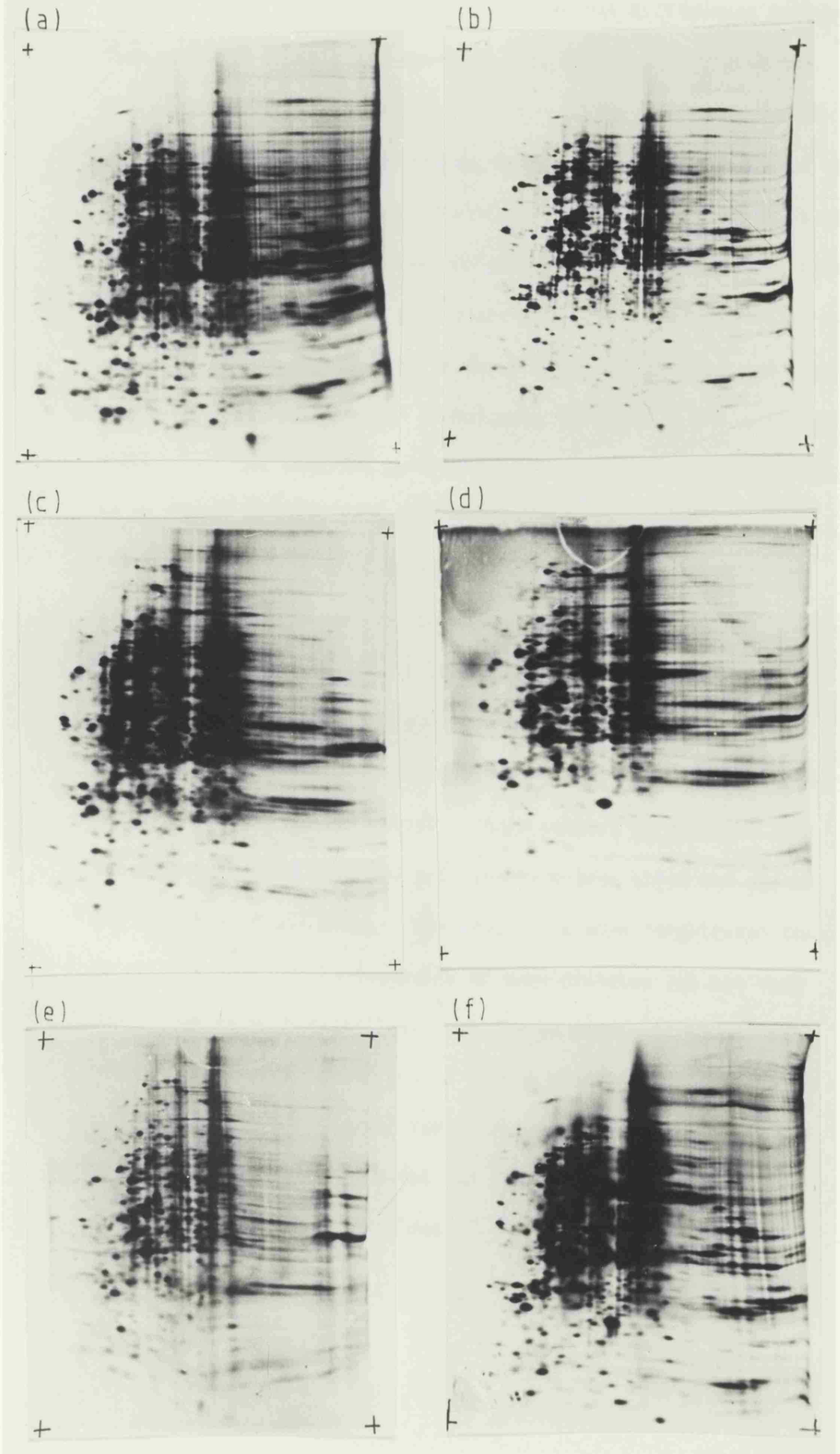
Fig.6.4. 2D gel pattern of radiolabelled protein from cells incubated in various conditions. Extracted proteins were separated by IEF on a pH gradient of 3.5-10.0 and 5-15% SDS-PAGE.

a) HMy2 cells incubated with ^{35}S-Met(12.5µCi) for 4 hr.
b) HMy2 cells incubated with ^{35}S-Met(12.5µCi) for 24 hr.
c) RPMI1788 cells incubated with ^{35}S-Met(50µCi) for 4 hr, in medium with FCS.
d) RPMI1788 cells incubated with ^{35}S-Met(12.5µCi) for 4 hr, in medium without FCS.
e) RPMI1788 cells incubated with ^{35}S-Met(15µCi) for 4 hr
f) RPMI1788 cells incubated with ^{35}S-Met(50µCi) for 4 hr.

c and f) Extracted protein were isoelectrofocussed on different day.

Exposure: 140,000-736,000 cpm; 6-15 days; -70^{o}C.

Fig. 6.4

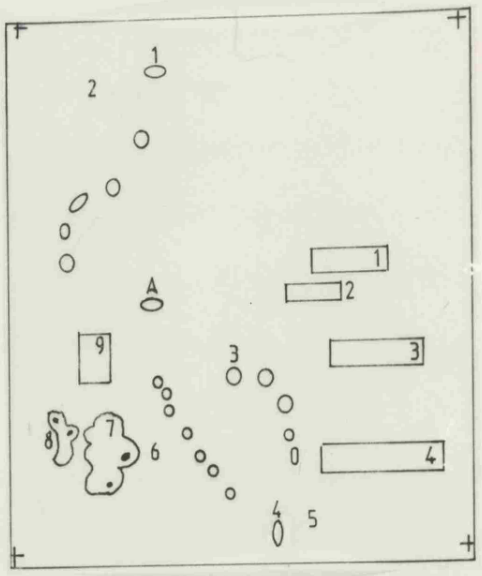


The 2D gel patterns of the cell lines (RPMI 1788, RPMI 8226, HMy2 and K562) were similar to each other but differences between them could be detected as shown in Fig. 6.5. together with the drawing line of some interested areas. The spot A on these 2D gel patterns was characterized as the actin from its M.W. of 42K and pI value of 5.25 ± 0.30 (Garrels and Gibson,1979). The common areas among these 4 cell lines were box 2, 3, 4, 7, 8 and 9. K562 cells showed a difference from other cell lines in the absence of labelled proteins in the area of box 1 and spot numbers 3 and 4 and in the presence of labelled proteins in the area of box 5 and spot number 1. The labelled protein at spot numbers 2 and 3 appeared to be absent in RPMI 1788 and HMy2 cells, respectively. The labelled protein in area 6 was present only in RPMI 8226. In some areas, such as spot numbers 2 and 3, there were quantitative differences among these cell lines.

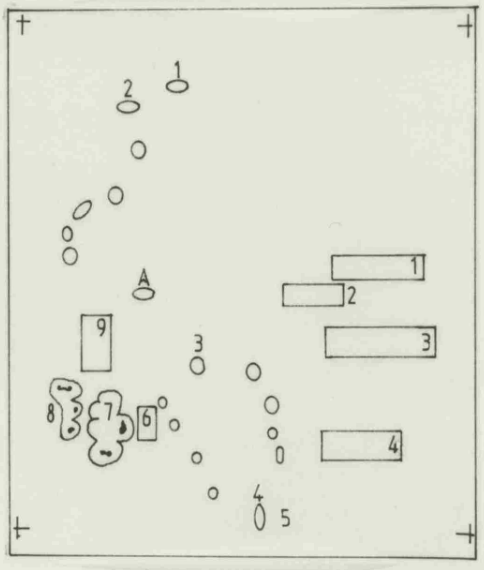
The results of 2D gel electrophoresis show that these cell lines have similar cellular products and they appeared to be different mainly in the amount of each product in relation to the others. More than 100 proteins have been shown and spread out over the 2D gel area. The result was more complicated than 1D gel patterns and the intensity of some proteins was not very consistent in each cell line. It may be difficult to use this method for showing the effect of ANAs on protein synthesis in intact cells. For general investigation, only the efficiency of cells to incorporate ^{35}S-Met and single dimensional-gel electrophoresis were determined.

Fig.6.5

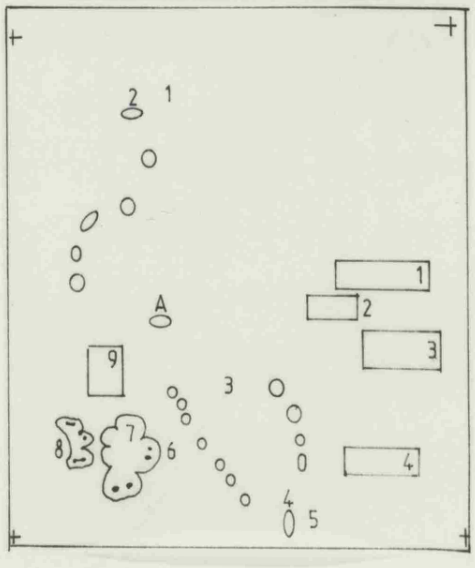
a.



b.



c.



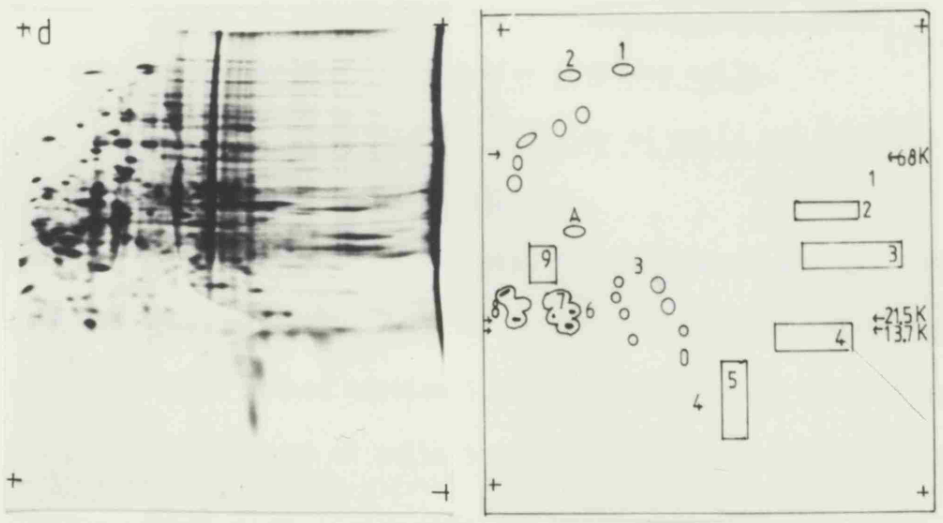


Fig. 6.5. 2D gel pattern of extracted protein from cell lines. The extracted proteins were focussed on gel at pH gradient 3.5-10.0.and run on 5-15% SDS-PAGE.

a) RPMI1788($5x10^6$)cells ($25\mu Ci, ^{35}S$-Met)4hr incubation in medium with FCS.
b) RPMI8226($5.2x10^6$)cells($25\mu Ci, ^{35}S$-Met)4hr incubation in medium with FCS.
c) HMy2($5.7x10^6$)cells ($25\mu Ci, ^{35}S$-Met) 4hr incubation in medium with FCS.
d) K562($2.5x10^6$)cells ($12.5\mu Ci, ^{35}S$-Met) 4hr incubation in medium with FCS.

Exposure: 156,000 cpm; 6-12 days; $-70^{o}C$.

Line drawing of 2D gel of RPMI1788, RPMI8226, HMy2, and K562 were shown as a box area (1-9) or spots (1-4) and the actin position is near the acid end (A).

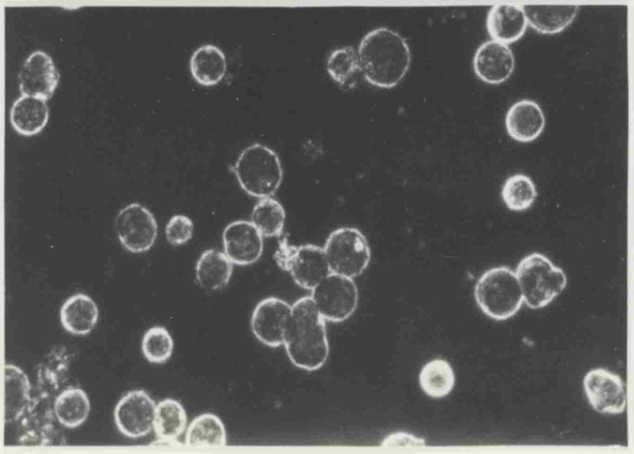


Fig. 6.7. Fluorescent micrograph of RPMI1788 cells incubated at $37^{o}C$ with liposome containing FITC-IgG.

6.2.2. Effect of ANAson Protein Synthesis in Intact Cells.

6.2.2.a. Using liposomes to transfer ANAsinto cells.

i). Effect of liposomes on viability of cells and incorporation of ^{35}S-Met by cells.

Empty liposomes filled with PBS were incubated with RPMI 1788 and HMy2 cells with viabilitiesof90.5% and 95.4%, respectively, as described in method section 6.1.2.a. The control was set up using the same amount of cells but incubated in PBS without liposomes. After 18 hr incubation, the cells were labelled with ^{35}S-Met for 3 hr. The viability of cells was reduced to 83.4% for RPMI 1788 cells and 86.1% for HMy2 cells when cells were treated with liposomes. The cell viability in controls was 87.9% for RPMI 1788 cells and 90.6% for HMy2 cells.

The incorporation of ^{35}S-Met was dependent on incubation time and showed no difference between liposome-treated and untreated cells (Fig 6.6.). After 3 hr incubation with ^{35}S-Met, the incorporation of ^{35}S-Met was 3.24 and 4.21 cpm/cell for RPMI 1788 and HMy2 cells, respectively. The control gave 3.59 cpm/cell for RPMI 1788 cells and 4.57 cpm/cell for HMy2 cells. There was a slight effect on viability of the cells and incorporation of ^{35}S-Met by cells when treated with liposomes.

ii). Efficiency of liposomes to entrap and transfer macromolecules into cells.

Using cytochrome c.

Cytochrome c was used to determine the efficiency of liposomes to entrap macromolecules. Various ratios of phosphatidyl choline(PC)/cholesterol were used. The results are shown in Table 6.2. About 12.5 - 21.8% of cytochrome c was entrapped into liposomes prepared from PC and cholesterol at ratios of 8:1, 16:1, and 24:1.

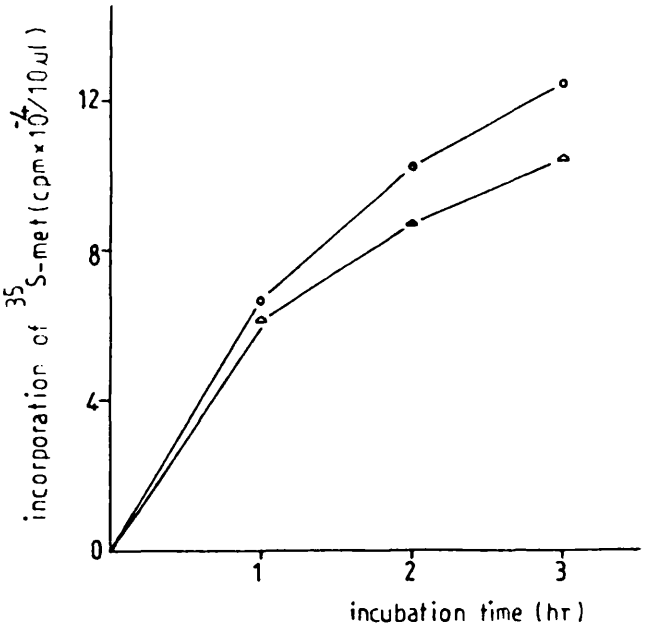


Fig. 6.6 Effect of liposomes on the incorporation of ^{35}S-Met by HMy2 cells.
Control: $2x10^6$ cells were incubated with PBS(o—o).
Experiment: $2x10^6$ cells were incubated with 1ml of liposomes containing PBS(△—△).

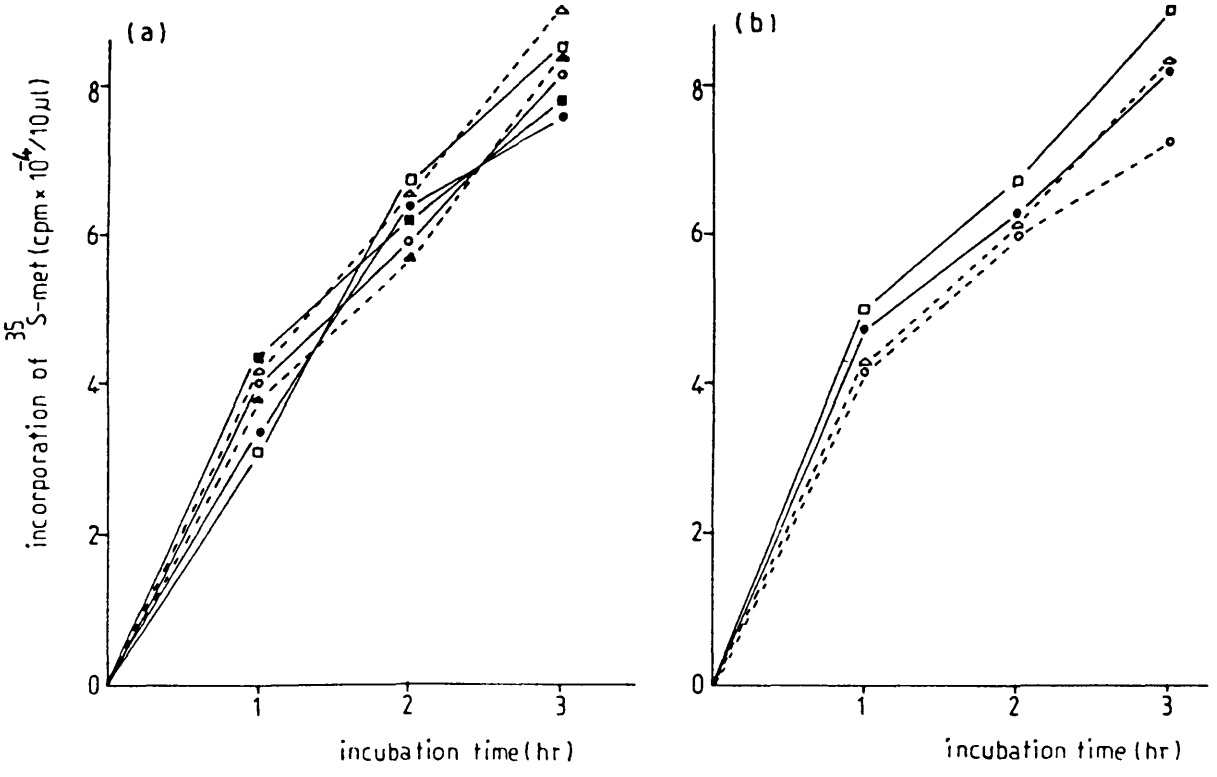


Fig. 6.8. Effect of ANAs transferred into cells by liposomes on protein synthesis in RPMI1788(a) and K562(b) cells. Cells were incubated with liposomes filled with

a) PBS, 2hr (□—□) and 18hr(■—■) incubation; normal IgG(M,K), 2hr(●—●) and 18hr(o—o) incubation; antiRo(Frayne), 2hr(▲---▲) and 18hr(△---△) incubation

b) PBS(□—□); normal IgG(NG)(●—●); antiRo(Frayne)(△---△); antiRNP(Windsor) (o---o), for 18 hr incubation.

Table 6.2. Encapsulation of cytochrome c in liposomes.

Ratio of PC/cholesterol	cytochrome c added (A_{414} units)	cytochrome c in liposomes (A_{414} units)	cytochrome c entrapped in liposomes(%)
8:1	30	3.35-4.16	12.50±1.35 (n=3)
16:1	30	5.42-5.90	18.85±0.80 (n=3)
24:1	30	5.72-7.38	21.82±2.77 (n=3)

Table 6.3. Preparation of ^{125}I-IgG-liposomes and transfer of ^{125}I-IgG from liposomes to intact cells.

Cell line	Experiment sample				Control sample	
	^{125}I-IgG entrapped in liposomes(%)	^{125}I-IgG uptake by cells(%) 1	2	recovery of radioactivity(%)	^{125}I-IgG uptake by cells(%)	recovery of radioactivity(%)
RPMI1788 (4×10^6) (n=2)	36.02±5.93	2.33±0.71	6.21±1.03	73.55±5.76	1.38±0.66	88.23±6.79
RPMI1788 (2×10^6) (n=5)	33.03±4.26	2.16±1.26	7.16±3.59	79.79±3.76	0.76±0.29	91.95±1.31
K562 (2×10^6) (n=3)	30.85±4.51	2.55±1.06	7.59±3.26	78.75±5.23	0.61±0.09	92.68±1.16

Note: 1 and 2 were percent binding of ^{125}I-IgG with cells which were calculated from total added radioactivity and total encapsulated radioactivity in liposomes, respectively.

This value was less different between liposomes prepared from PC/cholesterol at ratios of 16:1 and 24:1. The ratio of PC/cholesterol at 16:1 was chosen for preparation of liposomes.

Using ^{125}I-labelled IgG.

^{125}I-IgG was prepared from sheep IgG and had a protein concentration of 0.146µg/ml. ^{125}I-IgG was counted in a gamma counter for calculation of the results. 2-4µl of ^{125}I-IgG with 0.64mg of normal human IgG (as carrier) was used to prepare 1ml of liposomes. 2 X 10^6 cells were incubated with 0.5ml of liposomes for 2 hr, at 37°C. The results are shown in Table 6.3.

About 30% to 36% of total ^{125}I-IgG was entrapped in liposomes. After incubation of these liposomes with RPMI 1788 and K562 cells, the uptake of ^{125}I-IgG into cell was 2.2-2.6% and 6.2-7.5% of total cpm of ^{125}I-IgG added and ^{125}I-IgG in liposomes, respectively. This value was only 0.6-1.4% in the control in which cells were incubated with empty liposomes and free of ^{125}I-IgG. These results showed that liposomes can transfer IgG into cells, though with a low efficiency (about 1-2%).

Using FITC-IgG.

The liposomes loaded with FITC-IgG were incubated with RPMI 1788 cells, at room temperature, for 30 min and incubation was continued at 37°C, for 2 hr, after dilution with fresh medium. The cells were washed three times with PBS, fixed on a microscope slide and checked for fluorescent cells under UV light with a Leitz Orthoplan microscope. Most of the cells had fluorescence around the surface and some of them had fluorescence throughout the cell (Fig.6.7.). This experiment showed that, there was a binding of

Table 6.4. Viability and ^{35}S-Met incorporation into cells(RPMI1788 and K562) after incubation with liposomes containing PBS, normal IgG, or ANAs.

Cell line	liposomes contents	incubation time(hr)	viability (%)	^{35}S-Met incorporation (cpm/cell)	^{35}S-Met incorporation (cpm/µl medium)	recovery of extraction(%)
I.RPMI1788 ($2x10^6$)	PBS(n=2)	2	86.93±0.98	4.12±0.31	439.33±85.69	67.46±8.27
"	normal IgG(M,K) 0.8mg/ml,(n=2)	2	82.35±0.98	4.22±0.28	533.50±38.82	55.08±3.26
"	antiRo(Frayne) 0.8mg/ml,(n=2)	2	90.24±0.29	4.37±0.53	539.97±67.71	57.86±6.10
II.RPMI1788 ($2x10^6$)	PBS(n=4)	18	75.78±0.35	4.32±0.41	385.43±80.62	56.09±12.91
"	normal IgG(M,K) 0.8mg/ml,(n=4)	18	80.03±3.37	4.62±0.35	399.04±35.57	62.76±14.51
"	antiRo(Frayne) 0.8mg/ml,(n=2)	18	77.89±2.89	4.35±0.22	527.10±43.20	59.26±10.03
"	antiRo(Frayne) 4mg/ml,(n=2)	18	77.31±0.86	5.13±0.04	417.58±61.10	54.39±3.99
"	antiRNP(Windsor) 0.8mg/ml,(n=2)	18	78.10±0.69	4.81±0.04	337.49±49.95	49.92±12.36
III.K562 ($2x10^6$)	PBS(n=2)	18	89.06±3.35	4.55±0.06	397.08±30.52	60.56±1.96
"	normal IgG(NG) 1.2mg/ml,(n=2)	18	92.28±1.42	4.23±0.16	423.87±88.13	60.18±2.01
"	antiRo(Frayne) 4mg/ml,(n=2)	18	92.56±0.89	3.91±0.13	415.39±62.47	52.21±3.70
"	antiRNP(Windsor) 0.8mg/ml,(n=2)	18	94.16±2.07	4.02±0.07	299.37±31.59	57.52±2.20

The cells were incubated with liposomes for 2 and 18 hr before labelling. The incorporation of ^{35}S-Met into cells and culture medium was determined at 3hr labelling. The recovery of extraction was calculated as shown in Table 6.1. All the values were the mean values with standard deviation.

liposomes to the cells but could not show the transfer of FITC-IgG into cells. The FITC-IgG may transfer into cells at low concentration and this method may not be sensitive enough to detect it.

iii). Effect of ANAs on protein synthesis in intact cells.

Liposomes were used to transfer ANAs and normal IgG into cells. The control was cells treated with empty liposomes (loaded with PBS) or with liposomes containing normal human IgG at the same concentration as ANAs. After incubation with liposomes for 2 hr or 18 hr, the cells were washed and then labelled with ^{35}S-Met. The incorporation of ^{35}S-Met was determined at 1, 2 and 3 hr of incubation and either cell lysate or medium were analyzed on gel electrophoresis.

About 2 X 10^6 RPMI 1788 cells, with viabilities of 86-95% were incubated with 0.5ml of liposomes. Duplicates of each sample were compared to controls in each experiment. With antiRo (Frayne) at concentrations of 0.4mg or 2.0mg in 0.5ml liposomes, the incorporation of ^{35}S-Met by cells was similar to control during 3 hr incubation, after either 2 hr or 18 hr of incubation of cells with empty liposomes or liposomes containing normal IgG (Keowan at 0.4mg in 0.5ml liposome) (Fig 6.8.a.). This result was confirmed by the incorporation of ^{35}S-Met by cells and the incorporation into secreted protein which showed insignificant differences between control and ANAs (Table 6.4). The viability of cells was reduced to 82-90% and 75-80% after 2 hr and 18 hr incubation with liposomes respectively. The same result was given when using antiRNP (M.Windsor) antibodies, there was no change in incorporation of radioactivity after incubation of cells with liposomes containing

antiRNP antibodies (Windsor), (0.4mg/0.5ml liposome) for 18 hr (Table 6.4. and Fig 6.8.a).

The labelled proteins from these cells were analyzed on SDS-PAGE and their fluorograms are shown in Fig. 6.9.a, b, and c. The protein profiles were similar for controls and with ANAs, no bands weremissing and the intensity of each band was not significantly different between controls and with ANAs.Similar results were given in both extracted protein from cells (Fig.6.9 a. and b) and medium (Fig. 6.9.c. lane 1-7). There was no effect of ANAson protein synthesis in RPMI 1788 cells following transfer into cells by liposomes.

K 562 cells (about 2 X 10^6 cells) were incubated with 0.5ml liposomes prepared using antiRo (Frayne) 2mg, antiRNP (Windsor) 0.55mg, normal IgG (NG) 0.6mg and PBS, respectively, for 18 hr before labelling with ^{35}S-Met. There was no difference in incorporation of ^{35}S-Met between controls and with ANAs (Fig.6.8.b). The cell viability was slightly decreased after 18 hr incubation with liposomes (from 95% to 89-94%). The amount of radioactivity in medium, cells and the recovery of radioactivity in extracted protein are shown in Table 6.4. By gel analysis of extracted protein and medium, no effect of ANAson protein synthesis could be detected in these K562 cells (Fig.6.9.c. and d). The protein profile was the same in either control or ANA-treated cells. The fluorogram of labelled protein from RPMI 1788 cells treated with liposomes containing ANAsor normal IgG showed no difference in intensity of each band between controls and with ANAs. Quantitative determination of each band was achieved by scanning the X-ray film of the fluorogram at 540 nm and calculating the area under the peak with a fixed arm planimeter. The scanning profile is shown in

Fig. 6.9. Characterization of the proteins synthesized in RPMI1788 and K562 cells after incubation with liposomes by electrophoresis on 5-15% SDS-PAGE.

a) RPMI1788 cells incubated with liposomes containing PBS, normal IgG and ANAs for 2 and 18 hr. All the samples were extracted proteins from cells.

lane 1: PBS in liposome , 2hr incubation
" 2: normal IgG(M.K) in liposome(duplicated samples),2hr incubation
" 3: normal IgG(M.K) " " (" "),2hr "
" 4: antiRo(Frayne) " " (" "),2hr "
" 5: antiRo(Frayne) " " (" "),2hr "
" 6: standard proteins: ^{14}C-BSA, ^{14}C-trypsin inhibitor, ^{14}C-RNase
" 7: normal IgG(M.K) in liposome,2hr incubation
" 8: PBS in liposome,18 hr incubation
" 9: normal IgG(M.K) in liposome(duplicated samples),18hr incubation
" 10: normal IgG(M.K) " " (" "),18hr "
" 11: antiRo(Frayne) " " (" "),18hr "
" 12: antiRo(Frayne) " " (" "),18hr "

Exposure: 205,000 cpm; 1 day; -70°C.

b) RPMI1788 cells incubated with liposomes containing PBS, normal IgG and ANAs for 18 hr. All the samples were extracted proteins from cells.

lane 1: PBS in liposome (from duplicated samples)
" 2: PBS " " (" " ")
" 3: normal IgG(M.K) in liposome(from duplicated samples)
" 4: normal IgG(M.K) " " (" " ")
" 5: antiRo(Frayne) " " (" " ")
" 6: antiRo(Frayne) " " (" " ")
" 7: antiRNP(Windsor) " " (" " ")
" 8: antiRNP(Windsor) " " (" " ")
" 9: extracted protein of cells that did not treat with liposomes
" 10: " " " " " " " " " "
" 11: normal IgG(M.K) in liposome,2hr incubation
" 12: normal IgG(M.K) " " ,18hr "

Exposure: 200,000 cpm; 20 hr; -70°C.

c) Culture medium from RPMI1788 and K562 cells that incubated with liposomes containing PBS, normal IgG and ANAs for 2 and 18 hr.

lane 1: PBS in liposome with RPMI1788 cells,18hr incubation
" 2: normal IgG(M.K) in liposome with RPMI1788 cells,18hr incubation
" 3: antiRNP(Windsor) " " " " " ,18hr "
" 4: antiRo(Frayne) " " " " " ,18hr "
" 5: PBS in liposome with RPMI1788 cells,2hr incubation
" 6: normal IgG(M.K) in liposome with RPMI1788 cells,2hr incubation
" 7: antiRo(Frayne) " " " " " ,2hr "
" 8: standard proteins: ^{14}C-BSA, ^{14}C-trypsin inhibitor, ^{14}C-RNase
" 9: PBS in liposome with K562 cells,18hr incubation
" 10: normal IgG(M.K) in liposome with K562 cells,18hr incubation
" 11: antiRo(Frayne) " " " " " ,18hr "
" 12: antiRNP(Windsor) " " " " " ,18hr "

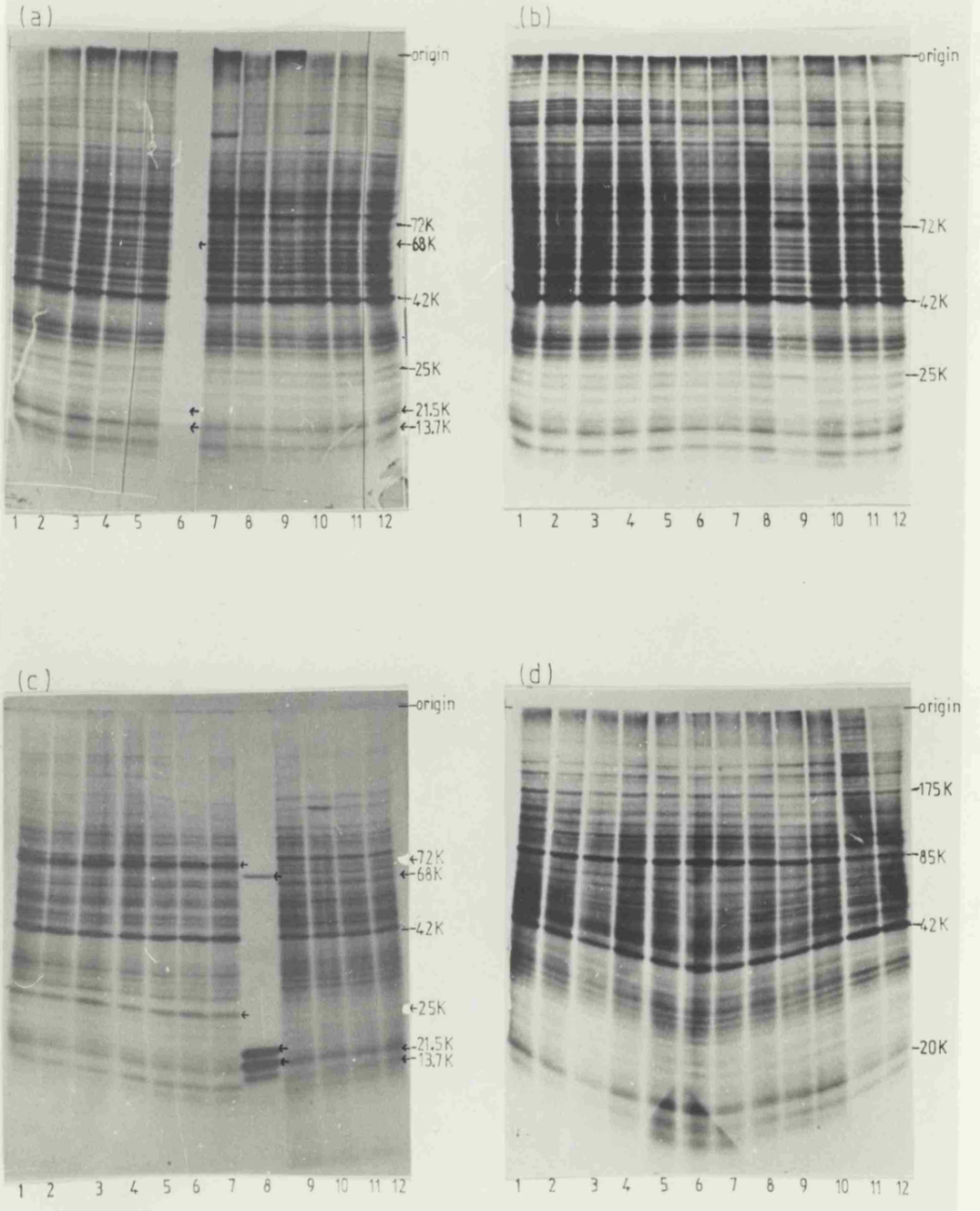
Exposure: 10,000 cpm; 15 days; -70°C.

d) K562 cells incubated with liposomes containing PBS, normal IgG, and ANAs for 18 hr. All samples were extracted proteins from cells.

lane 1: PBS in liposome(from duplicated samples)
" 2: PBS " " (" " ")
" 3: normal IgG(NG) in liposome(from duplicated samples)
" 4: normal IgG(NG) " " (" " ")
" 5: antiRo(Frayne) " " (" " ")
" 6: antiRo(Frayne) " " (" " ")
" 7: antiRNP(Windsor)" " (" " ")
" 8: antiRNP(Windsor)" " ("" " ")
" 9: control,K562 cells incubated in PBS for 2hr before radiolabelling
" 10: control, " " " " " 18hr " "
" 11: PBS in liposome with RPMI1788 cells,18hr incubation
" 12: PBS " " " " " ,2hr "

Exposure: 195,000 cpm; 20 hr; -70°C.

Fig.6.9


(a)
origin
72K
68K
42K
25K
21.5K
13.7K
1 2 3 4 5 6 7 8 9 10 11 12
(b)
origin
72K
42K
25K
1 2 3 4 5 6 7 8 9 10 11 12
(c)
origin
72K
68K
42K
25K
21.5K
13.7K
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(d)
origin
175K
85K
42K
20K
1 2 3 4 5 6 7 8 9 10 11 12

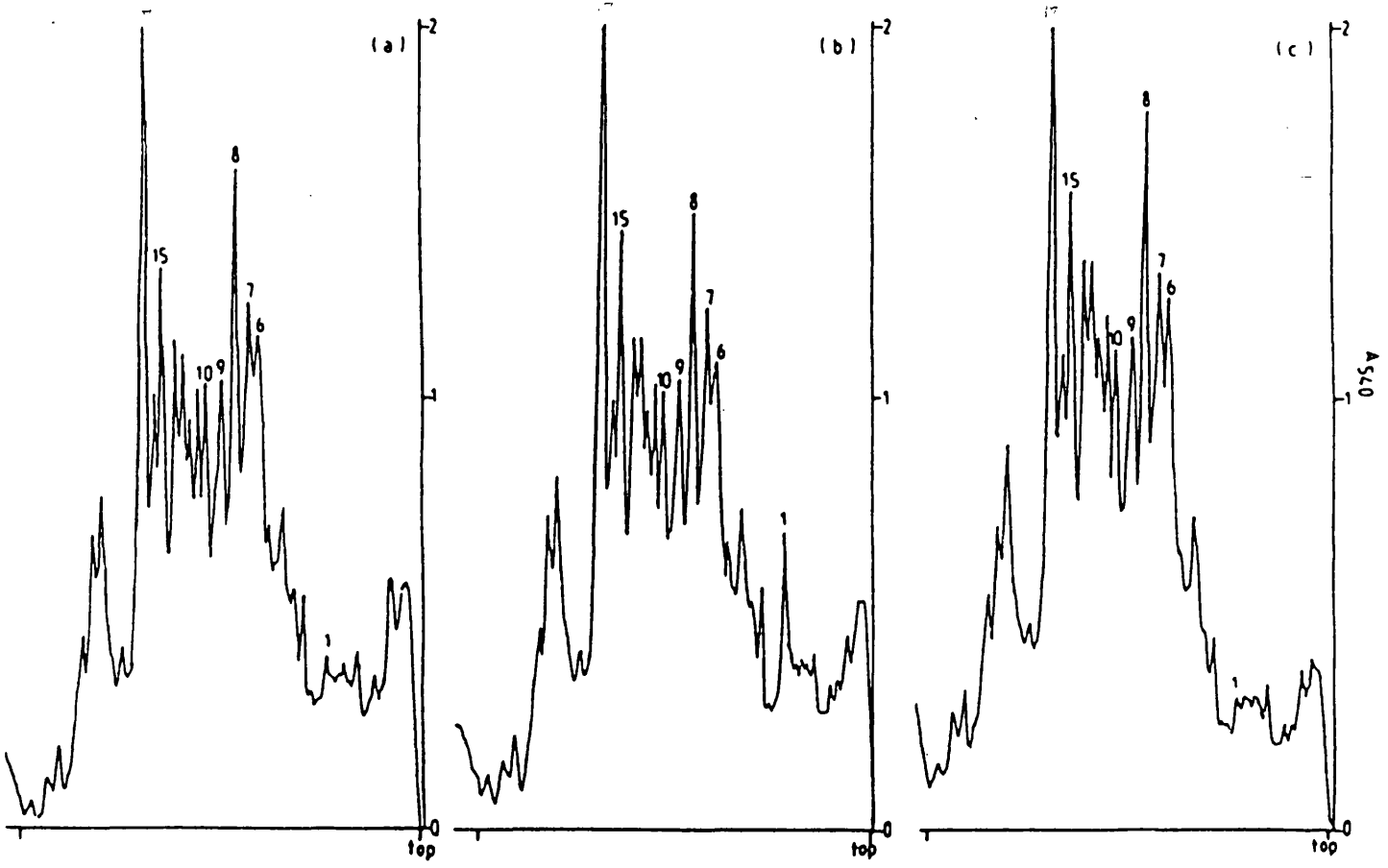


Fig. 6.10. Scanning profile of X-ray film of fluorogram from Fig. 6.9.b. at wavelength 540 nm. The RPMI1788 cells were incubated with liposomes containing PBS (a), normal IgG(M.K) (b) and antiRo(Frayne) (c) for 18 hr before radiolabelling protein with ^{35}S-Met. The labelled proteins were separated on 5-15% SDS-PAGE.

Table 6.5. Mean peak areasdetermined from scanning the profile of fluorogram of extracted protein from RPMI 1788 cells after treatment with liposomes containing PBS, normal IgG,or ANAs.

Sample	Peak area (arbitrary units)							
	peak 1 (M.W=203K)	peak 6 (M.W=108K)	peak 7 (M.W=97.5K)	peak 8 (M.W=85.5K)	peak 9 (M.W=74K)	peak 10 (M.W=68K)	peak 15 (M.W=48K)	peak 16 (M.W=46K)
1.PBS (n=4)	0.10±0.04	0.56±0.08	0.58±0.12	0.71±0.12	0.72±0.06	0.53±0.08	0.65±0.07	0.46±0.07
2.normal IgG(M.K) (n=7)	0.15±0.11	0.49±0.09	0.40±0.04	0.60±0.09	0.73±0.09	0.47±0.11	0.59±0.10	0.41±0.07
3 antiRo (Frayne) (n=6)	0.13±0.06	0.53±0.11	0.51±0.13	0.61±0.09	0.75±0.06	0.49±0.12	0.66±0.15	0.46±0.11

(n: represents replicate gel runs)

Fig 6.10. a, b and c. The average area of some peaks is shown in Table 6.5. This result confirmed that protein synthesis in cells was not affected after incubation with liposomes containing ANAs

iv). Effect of cycloheximide on protein synthesis in K562 cells.

The incorporation of radioactivity by K562 cells (1×10^6) was inhibited after incubation with cycloheximide at various concentrations for 2 hr and followed by washing twice before labelling with ^{35}S-Met. The inhibition was dependent on the concentration of cycloheximide (Fig 6.11.a). Inhibition was 19% for cycloheximide at 120µg and increased to 68.13% at 500µg. The inhibition was as high as 94.27% when the cells were labelled with ^{35}S-Met in the presence of 100µg of cycloheximide (Fig 6.11.a). This result showed that an inhibitory effect can be detected by reduced incorporation of radioactivity using cycloheximide, a small molecule which can easily diffuse in and out of the cells.

When K562 (1×10^6) cells were incubated with liposomes containing cycloheximide, the percentage inhibition was found to be 18.3%. If 35% of cycloheximide was entrapped in the liposomes and only 2% was transferred into cells (from the data using ^{125}I-IgG in section 6.2.2.a (ii)), the total amount of cycloheximide in the cells was about 100µg. This amount of cycloheximide (120µg) will affect the incorporation of radioactivity, showing 19% inhibition in cells treated with this amount of free cycloheximide. The result of this experiment showed that cycloheximide can transfer into the cells by using liposomes in a low concentration which results in a small effect on protein synthesis.

This effect of cycloheximide on protein synthesis in K562 cells was further investigated by gel analysis. In Fig

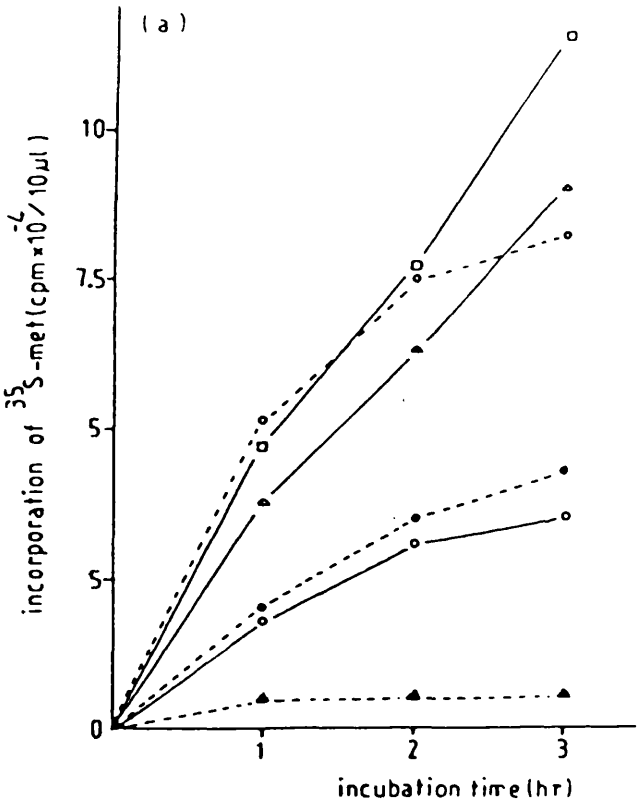


Fig. 6.11. a) Inhibition of ^{35}S-met incorporation in K562 cells by cycloheximide.

Cells incubated with PBS(▫—▫)

Cells incubated with liposomes containing cycloheximide(▵—▵); %inhibition = 18.30

Cells incubated with cycloheximide and washed before labelling with ^{35}S-Met

-at 125µg(o---o); %inhibition=19.00
-at 360µg(•---•); " =61.61
-at 500µg(o—o); " =68.13

Cells incubated with ^{35}S-Met in the presence of 100µg of cycloheximide (▲----▲); %inhibition=94.27.

(%inhibition calculated at 3 hr incubation).

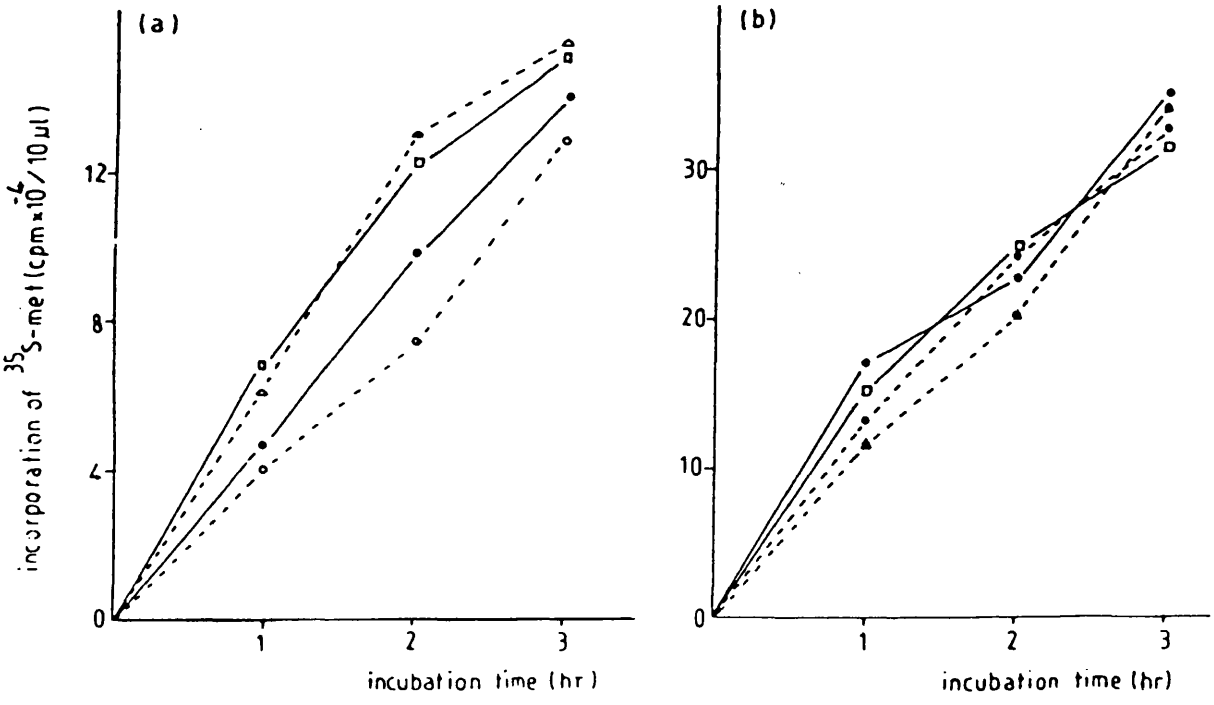
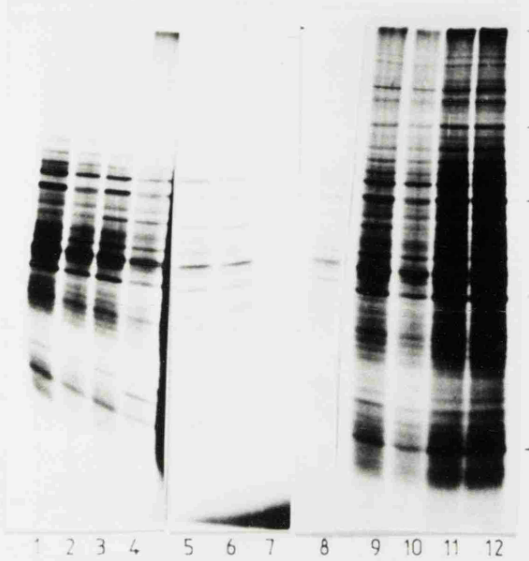


Fig. 6.12. a and b) Effect of ANAs transfered into cells by red cell ghosts on protein synthesis in K562 cells(a) and X63 mouse cells(b). Labelling of cells was carried out in 1ml medium without met.

a) the K562 cells were fused with red cell ghosts containing PBS(▫—▫), normal IgG(NG)0.6mg(•—•), antiRo(Frayne)2mg(▵---▵),antiRNP(Windsor) 0.55mg(o---o).

b) the X63 mouse cells fused with red cell ghosts containing PBS(▫—▫), normal IgG(NG)0.6mg(•—•), antiRNP(Windsor)0.55mg(▲---▲), antiRNP (Swindell)0.6mg(•---•).

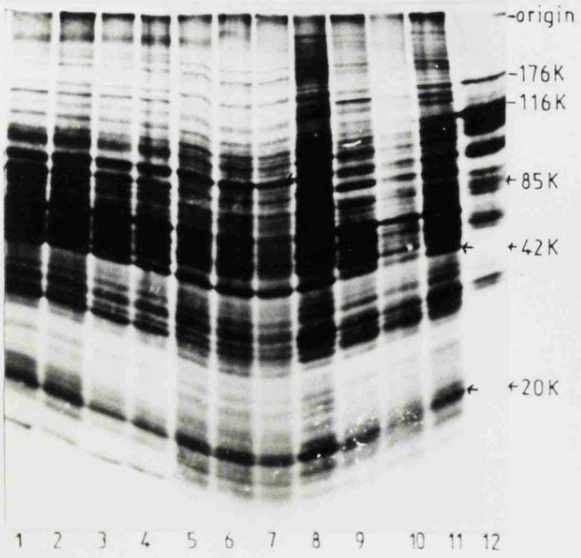
Fig. 6.11. b) Fluorogram of labelled protein from K562 cells incubated with cycloheximide. The samples were medium and extracted proteins from cells and were loaded at either the same volume or the same amount of radioactivity and run on 5-15% SDS-PAGE.



lane 1: EP, with 120µg cycloheximide
" 2: EP, " 360µg "
" 3: EP, " 500µg "
" 4: EP, " 100µg "
while radiolabelling
" 5: M, with 120µg cycloheximide
" 6: M, " 360µg "
" 7: M, " 100µg "
while radiolabelling
" 8: EP, with 100µg cycloheximide
while radiolabelling
" 9: EP, with 500µg cycloheximide
" 10: EP, " 360µg "
" 11: EP, " 120µg "
" 12: EP, " PBS "

Exposure 10,000-276,000 cpm;
18 hr & 12 days; -70°C
(EP=extracted protein; M=medium)
lane 1-7 the same cpm(10,000)
" 8-12 the same vol(2µl)

Fig. 6.12. c) Fluorographic image of protein synthesized in K562 cells treated with liposome and red cell ghosts containing PBS, normal IgG and ANAs and protein synthesized in X63 mouse cells after treatment with red cell ghosts containing PBS, normal IgG and ANAs. All samples were extracted protein containing the same amount of radioactivity.



lane 1: K562 with RCG(PBS)
" 2: " with RCG(normal IgG)
" 3: " with RCG(antiRo)
" 4: " with RCG(antiRNP)
" 5: " with liposome(PBS)
" 6: " with liposome(antiRo)
" 7: " with liposome(antiRNP)
" 8: X63 incubated with PBS
" 9: " with RCG(PBS)
" 10: " with RCG(normal IgG)
" 11: " with RCG(antiRo)
" 12: TMV proteins as standard

Exposure: 190,000 cpm; 20hr; -70°C.

(RCG=red cell ghost; normal IgG (NG); antiRo(Frayne); antiRNP (Windsor)).

6.11.b. the inhibitory effect is clearly shown and all types of proteins were synthesized in reduced amount. The inhibition did not affect any particular type of protein. At lower percent inhibition (19%), the intensity of protein bands was similar to the control (Fig.6.11.b. lane 1,11 and12). Therefore, the effect was not easily shown.

The results of this experiment helped to explain the difficulty in using liposomes for transfer of macromolecules into cells and the difficulty in showing the inhibitory effect on cellular protein synthesis. Because the synthesis of all proteins was inhibited, the percentage inhibition should be quite high to show a difference in gel patterns.

Therefore, it was quite difficult to show an inhibitory effect on protein synthesis by ANAs transferred into cells in low concentration by liposomes. The cells require a rather high amount of ANAs to show high percentage inhibition and this technique(using liposomes)did not give high efficiency in transferring ANAs into cells. Only some cells bound to liposomes (showed by using FITC-IgG in section 6.2.2.a (ii)), and the percent incorporation of ^{35}S-Met was determined from all cells. The effect of ANAs on a small number of cells may not be detectable when ^{35}S-Met incorporation is measured for the entire cell population. Alternatively, although the liposomes were able to transfer ANAs into cells, the intracellular concentration might not be high enough to show any effect on protein synthesis in the cells as detected by incorporation of radioactivity and separation of labelled proteins on gel electrophoresis.

Table 6.6. Cell viability and ^{35}S-Met incorporation into cells(K562 and X63 mouse), treated with red cell ghosts containing PBS, normal IgG, or ANAs.

Cell line	red cell ghosts contents	viability (%)	^{35}S-Met incorporation (cpm/cell)	^{35}S-Met incorporation (cpm/µl medium)	recovery of extraction(%)
I.K562 ($4x10^6$)	PBS(n=2)	86.80±1.84	4.58±0.27	986.76±102.46	11.33±0.94
"	normal IgG(NG) (n=2)	83.61±5.04	4.78±0.32	1146.49±65.51	13.59±2.01
"	antiRo(Frayne) (n=2)	88.58±2.36	4.75±0.09	1103.36±108.69	14.09±2.51
"	antiRNP(Windsor) (n=2)	85.54±3.35	4.39±0.51	980.44±108.78	14.52±2.26
II.X63 ($4x10^6$)	PBS(n=2)	77.79±7.18	6.28±1.97	1130.93±146.63	14.24±1.98
"	normal IgG(NG) (n=2)	69.95±4.95	6.30±2.25	1035.62±45.37	12.37±0.79
"	antiRo(Frayne) (n=2)	80.17±6.06	6.41±1.95	1091.74±114.88	14.53±0.32
"	antiRNP(Swindell)	82.19	7.36	1206.62	14.21
"	antiRNP(Windsor)	74.11	6.14	929.45	17.81

The cells were incubated with red cell ghosts for 2 hr before labelling. The incorporation of ^{35}S-Met into cells and culture medium was determined at 3 hr labelling. The red cells were separated before extraction of protein from cells(K562 or X63) and the recovery of extraction was calculated as shown in Table 6.1. Most of the values were the mean value with standard deviation.

6.2.2.b. Using red cell ghosts to introduce ANAsinto cells.

Red cell ghosts were prepared by the pre-swell technique as described in method section 6.1.2.b. The red cells took up the loaded protein (normal IgG or ANAs)during the rapid hypotonic haemolysis. These red cell ghosts were fused to K562 or X63 cells using polyethylene glycol and loaded protein was transferred into the cells. X63-mouse cells were used because of their high percentage of fusions in the polyethylene glycol technique (Kearney et al.,1979).

The efficiency of this technique in transferring macromolecules into cell was checked by using ^{125}I-IgG. The X63-mouse cells were fused with red cell ghosts loaded with ^{125}I-IgG and incubated for 2 hr, at 37°C. About 20.5 ± 5.5% of total ^{125}I-IgG was found to load into red cell ghosts. Only 11.78 ± 0.44% of the amount of radioactivity in red cell ghosts was transferred to X63 mouse cells and the amount of radioactivity transferred into the cells was approximately 0.06 ± 0.02% of total radioactivity (calculated from the radioactivity in supernatant after lysis of the cells).

For controls, the X63 cells were fused with red cell ghosts prepared in the presence of PBS or normal IgG (NG),0.6mg and for ANA transfer; antiRNP (Windsor),0.55mg and antiRo (Frayne),2mg were used. K562 cells and X63 mouse cells were fused with these red cell ghosts, incubated for 2 hr, washed and labelled with ^{35}S-Met (as described in method section 6.1.2.b). The viability of cells was reduced from 85-95% to 83-88% for K562 cells and to 74-82% for X63 mouse cells after 2 hr incubation with red cell ghosts (Table 6.6.). The incorporation of ^{35}S-Met was not significantly different between controls and for ANAs in either K562 or X63 mouse cell

(Fig 6.12. a and b). The incorporation of radioactivity per cell, and the total radioactivity in cells and medium from these cells were determined and are shown in Table 6.6.

Red cell ghosts were removed before extraction of protein from cells (as described in method section 6.1.2.b). The recovery of cells was 60-67%. These extracted proteins were analyzed by gel electrophoresis and the fluorogram is shown in Fig 6.12.c. No qualitative or quantitative differences between synthesized proteins was seen between the cells incubated with normal IgG or PBS and with ANAs in K562 cells or X63 mouse cells.

6.2.2.c. Antibody penetration of cells through Fcγ receptors.

Alarcon-Segovia et al. (1978) have shown that ANAs can penetrate viable cells through Fcγ receptors. They incubated 1 X 10^6 mononuclear cells from normal subjects with 4.2mg of FITC-antiRNP antibody (which had a titre of 1:1,048,576), for 1 hr at 37°C. About 11%, 19% and 6% of viable cells showed membrane fluorescence, nuclear fluorescence, and cytoplasmic fluorescence, respectively. This method was used to transfer ANAs into cells in this study, but the concentration of ANAs used was lower. The highest amount of ANAs was 2.0mg with 1 X 10^6 cells (antiRo (Frayne)).

K562 cells were chosen for use in this experiment since the mean percentage of Fcγ receptors on these cells was 67.25±3.11% (4 experiments) whereas RPMI 1788, HMy2 and RPMI 8226 cells had 19.00 ± 3.34%, 11.75 ± 4.76%, and 9.5± 3.84%, respectively. Fcγ receptors were determined using the rosette assay as described in method section 2.2.1.d.

K562 cells, (about 1 X 10^6 cells) were incubated for 1 hr at 37°C at high concentration in PBS or with normal IgG(M.El-Naggar,

Table. 6.7. Cell viability, ^{35}S-Met incorporation into cells and culture medium and recovery of extraction on incubation of K562 cells with PBS, normal IgG and ANAs.

Cell line	incubated with	incubation time(hr)	viability (%)	^{35}S-Met incorporation (cpm/cell)	^{35}S-Met incorporation (cpm/µl medium)	recovery of extraction(%)
I.K562 ($1x10^6$)	PBS(n=4)	2	90.88±2.79	3.76±0.29	135.68±46.36	48.93±7.49
"	normal IgG(M,K) at 1.6mg,(n=2)	2	94.11±2.08	3.76±0.24	104.20±17.04	58.62±1.47
"	normal IgG(M,N) at 0.6mg,(n=2)	2	85.26±0.88	3.28±0.09	195.04±0.28	67.00±6.93
"	antiRNP(Windsor) at 1.1mg,(n=2)	2	90.65±2.90	3.76±0.07	98.20±12.59	38.82±0.05
"	antiRo(Frayne) at 0.45mg,(n=2)	2	89.92±0.27	3.38±0.05	204.43±10.37	48.62±2.26
"	antiRo(Frayne) at 2mg,(n=2)	2	94.37±1.28	3.20±0.06	179.15±3.27	44.06±5.24
"	antiSm/RNP(Summes) at 0.175mg,(n=2)	2	86.39±1.46	3.38±0.27	196.79±1.85	36.77±3.28
II.K562 ($1x10^6$)	PBS(n=2)	19	96.26±0.46	3.58±0.09	185.98±44.08	58.65±0.30
"	normal IgG(M,K) at 1.6mg,(n=2)	19	96.47±0.12	4.33±0.26	185.95±11.23	49.09±5.96
"	antiRNP(Windsor) at 1.1mg,(n=2)	19	95.28±1.61	4.23±0.21	161.78±17.36	51.91±6.55

The K562 cells were incubated with ANAs for 2 and 19 hr before labelling. The incorporation of ^{35}S-Met into cells and culture medium was determined at 3 hr labelling. The recovery of extraction was calculated as shown in Table 6.1. The mean value with standard deviation was presented.

0.6mg and Mc.Keowan,1.6mg) for controls and with ANAs(antiRo(Frayne), 0.45 and 2.0mg; antiRNP (Windsor),1.1mg; and antiSm/RNP (P.Summes) 0.175mg) for experiments. The cells were diluted with fresh medium and incubation continued for 2 hr or 19 hr before labelling proteins with ^{35}S-Met. Both 2 hr and 19 hr incubations of cells with ANAs gave the same results. There was no inhibitory effect on incorporation of radioactivity into cells during 3 hr incubation (Fig.6. 13. a and b). The viability of cells was not changed after incubation with ANAs. The incorporation of radioactivity per cell and total radioactivity in cells and medium were similar between controls and with ANAs. The average values are illustrated in Table 6.7.

The extracted protein from these K562 cells was further analyzed on SDS-PAGE as shown in Fig. 6.13.c. The result confirmed that there was no inhibitory effect on protein synthesis in K562 cells that were incubated with ANAs. The protein profile of these samples showed both quantitative and qualitative similarities between controls and ANA-treated cells.

6.2.2.d. Introduction of ANAsinto cells by permeabilization in the presence of ANAs.

i). Effect of permeabilization on the viability and incorporation of radioactivity into cells.

K562 cells were used in this experiment and it was found that the recovery of cells was 56-70% and the viability of cells was reduced from 90-95% to 75-85%, after permeabilization. The incorporation of radioactivity into cells was slightly lower than the control (untreated cells with equal number of cells). The incorporation of radioactivity was 8.36 ± 0.02 cpm/cell for control and 8.09 ± 0.08 cpm/cell for cells after permeabilization. Extracted proteins were analyzed on gel electrophoresis. In Fig.

Fig. 6.13. a and b) Incorporation of ^{35}S-Met by K562 cells after incubation with ANAs, PBS, and normal IgG for 2 hr(b) and 19 hr(a).

a) K562 cells($1x10^6$) incubated with PBS(o—o), normal IgG(M.K) 1.6mg(•—•) and antiRNP(Windsor) 1.1mg(▲---▲).

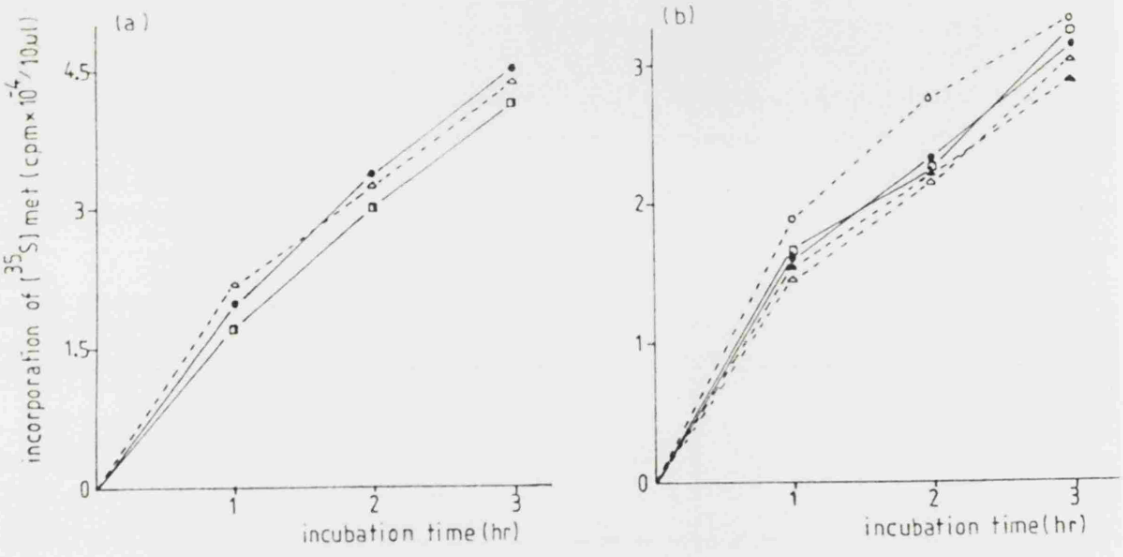
b) K562 cells($1x10^6$) incubated with PBS(o—o), normal IgG(M.N) 0.6mg(•—•), antiRo(Frayne) 0.45mg(▲---▲), antiRo(Frayne) 2.0mg(▲---▲) and antiSm/RNP(Summes) 0.175mg(o---o).

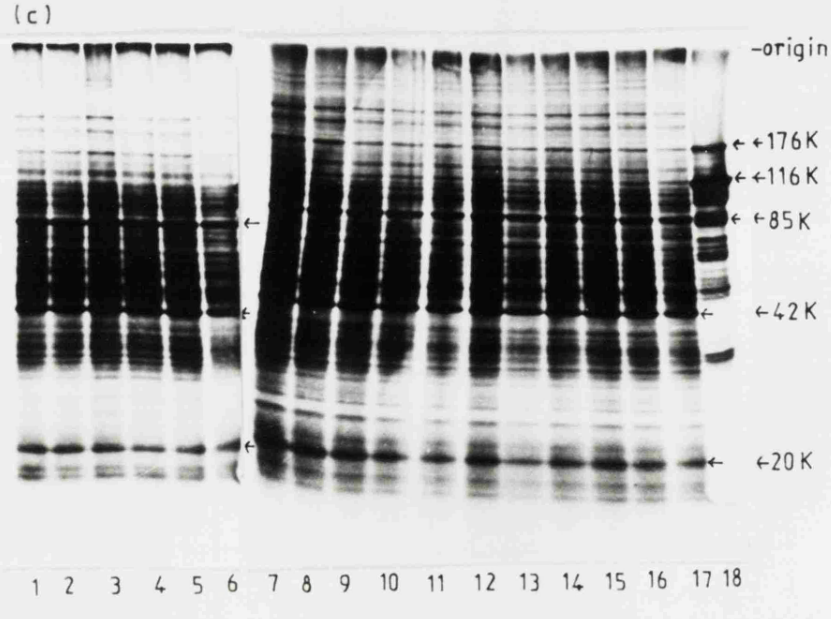
c) Characterization of proteins synthesized in K562 cells after incubation with ANAs for 2 hr and 19 hr. All samples were extracted proteins and the same amount of radioactivity from each sample was loaded onto 5-15% SDS-PAG.

lane 1-6: K562 cells incubated with ANAs for 19 hr.
" 7-17: K562 cells incubated with ANAs for 2 hr.
" 1: with PBS (duplicated samples)
" 2: " PBS (" ")
" 3: " normal IgG(M.K)(duplicated samples),1.6mg
" 4: " normal IgG(M.K)(" "),1.6mg
" 5: " antiRNP(Windsor)(" "),1.1mg
" 6: " antiRNP(Windsor)(" "),1.1mg
" 7: " PBS(duplicated samples)
" 8: " PBS(" ")
" 9: " normal IgG(M.N) (duplicated samples),0.6mg
" 10: " normal IgG(M.N) (" "),0.6mg
" 11: " antiRo(Frayne) (" "),0.45mg
" 12: " antiRo(Frayne) (" "),0.45mg
" 13: " antiRo(Frayne) (" "),2mg
" 14: " antiRo(Frayne) (" "),2mg
" 15: " antiSm/RNP(Summes)(" "),0.175mg
" 16: " antiSm/RNP(Summes)(" "),0.175mg
" 17: " PBS
" 18: TMV protein as standard

Exposure: 193,000 cpm; 20 hr; -70°C.

Fig. 6.13


(a)
incorporation of [35S] met (cpm × 10⁻⁴/10µl)
4.5
3
1.5
0
1
2
3
incubation time (hr)
(b)
3
2
1
0
1
2
3
incubation time (hr)


(c)
origin
176K
116K
85K
42K
20K
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

6.13. lane 1 and 2 and Fig6.15.c.lane 1 and 2, similar protein profiles are shown between control and permeabilized cells. There was no effect on protein synthesis in the cells after permeabilization procedure, the only effect was a reduction of the number of cells recovered.

ii). Efficiency of K562 cells in take up of macromolecules by permeabilization procedures.

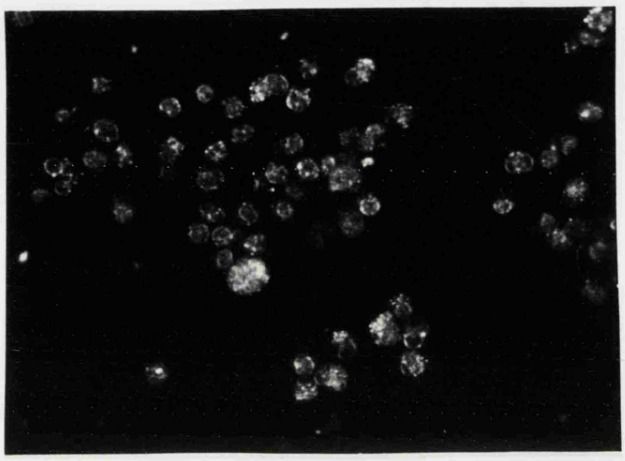
Using ^{125}I-labelled IgG.

K562 cells (2 X 10^6) were lysed in hypotonic solution containing ^{125}I-IgG (0.002µg) within 2 min and then resealed by adding hypertonic salt solution. The control was prepared by the same procedure, but the ^{125}I-IgG was added after resealing the cells. The cells were incubated at 37°C, for 1 hr, washed three times with PBS and radioactivity counted with a gamma counter. The amount of radioactivity inside the cell was determined from the supernatant after lysis of the cells. About 1.2-1.4% of total ^{125}I-IgG were detected with washed cells and only 0.5-0.7% of total ^{125}I-IgG were found inside the cells. In controls, the radioactivity of washed cells and inside cells was 0.5-0.69% and 0.16-0.3% of total ^{125}I-IgG, respectively. This result showed that ^{125}I-IgG was taken into cell but in low amount.

Using FITC-IgG.

The permeabilization procedure was done as described above, but K562 cells (2 X 10^6) were lysed in the presence of FITC-IgG (84µg). The cells were washed, fixed on slides and the fluorescence checked under UV light with a Leitz Orthoplan microscope. More than 70% of cells in the sample were fluorescent while this value was less than 20% in the control. Fig 6.14.

(a)



(b)

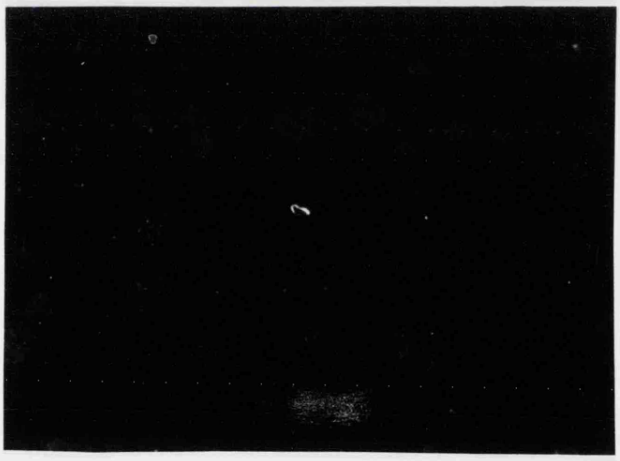


Fig. 6.14. Fluorescence micrograph of K562 cells permeabilized in the presence of FITC-IgG (a) and K562 cells incubated with FITC-IgG after permeabilization in PBS and resealing (b).

Table 6.8. Cell viability and ^{35}S-Met incorporation into cells(K562 and RPMI1788) after permeabilization in the presence of PBS, normal IgG, and ANAs.

cell line	permeabilized in	viability (%)	^{35}S-Met incorporation (cpm/cell)	^{35}S-Met incorporation (cpm/μl medium)	recovery of extraction(%)
I.K562 ($2x10^6$)	PBS(n=4)	85.77±1.74	4.83±0.31	561.48±123.19	66.62±3.17
"	normal IgG(NG) at 0.06mg,(n=2)	84.14±3.06	4.54±0.19	652.16±13.04	71.03±1.68
"	normal IgG(M.K) at 0.08mg,(n=2)	84.46±1.37	4.59±0.11	470.99±66.77	62.35±4.56
"	antiRo(Frayne) at 0.2mg,(n=2)	86.58±1.92	5.07±0.08	709.70±12.64	61.69±10.35
"	antiRo(Frayne) at 0.045mg,(n=2)	83.54±3.01	5.35±0.10	556.95±103.26	70.28±4.61
"	antiRNP(Windsor) at 0.05mg,(n=2)	86.45±2.16	5.38±0.07	568.43±130.03	62.33±2.82
II.RPMI1788 ($2x10^6$)	PBS(N=2)	71.65±1.01	3.23±0.22	286.25±60.69	40.43±10.01
"	normal IgG(NG) at 0.06mg,(n=2)	69.67±0.33	4.22±0.23	330.14±43.90	49.46±3.51
"	antiRo(Frayne) at 0.045mg(n=2)	73.28±0.78	3.51±0.06	382.78±73.55	60.19±2.91
"	antiRNP(Windsor) at 0.05mg,(n=2)	66.15±1.08	4.13±0.11	329.68±28.82	56.93±6.02

Cells were treated with PBS, normal IgG and ANAs which were introduced into cells by a permeabilization procedure. The incorporation of ^{35}S-Met into cells and culture medium was determined at 3 hr labelling. The recovery of extraction was calculated as shown in Table 6.1. All of the values were the mean values with standard deviations.

a and b shows typical staining patterns for experimental and control cells and demonstrates that fluorescent staining was less intense in the control. This result showed that most of the cells bound FITC-IgG.

Effect of ANAs on protein synthesis.

About 1.5-2.0 X 10^6 K562 or RPMI 1788 cells with viabilities more than 90% were lysed in hypotonic solution containing ANAs (antiRo (Frayne),0.2mg and AntiRNP (Windsor),0.05mg), PBS or normal IgG (NG, 0.06mg). After resealing, the cells were incubated for 3 hr at 37°C before labelling with ^{35}S-Met. In Fig 6.15 a and b, the results showed no difference in incorporation of radioactivity by both cell lines between controls and ANAs. The viability of cells was reduced to 83-87% for K562 cells and 66-71% for RPMI 1788 cells. Amounts of radioactivity per cell and total radioactivity in medium, including the recovery of extracted protein from cells were determined and are summarized in Table 6.8.

There was no inhibitory effect of ANAs on protein synthesis in either K562 or RPMI 1788 cells in this experiment and this result was also confirmed by gel analysis of extracted protein from these cells (Fig. 6.15. c and d). Similarity of protein profiles including intensity of each bands was seen between cells with ANAs, and control cells.

Fig. 6.15. Effect of ANAson protein synthesis in K562 and RPMI1788 cells. The cells were permeabilized in the presence of ANAs, normal IgG and PBS. After resealing, the cells were incubated with ^{35}S-Met.

a) K562 cells($2x10^6$) with PBS(□—□), normal IgG(M.K) 0.08mg(•—•), antiRo(Frayne)0.045mg(△---△) and antiRNP (Windsor)0.05mg(○---○).

b) RPMI1788 cells($2x10^6$) with PBS(□—□), normal IgG(NG) 0.06mg(•—•), antiRo(Frayne)0.045mg(△---△) and antiRNP (Windsor)0.05mg(○---○).

c and d) Characterization of synthesized protein in K562 cells(c) and RPMI1788 cells(d) after permeabilization in the solution containing PBS, normal IgG or ANAs by 5-15% SDS-PAGE/fluorography. All samples were extracted protein with the same amount of radioactivity.

c)

lane 1: in PBS(duplicated samples)
" 2: " PBS(" ")
" 3: " normal IgG(NG)0.06mg(duplicated samples)
" 4: " normal IgG(NG)0.06mg(" ")
" 5: " antiRo(Frayne)0.2mg (" ")
" 6: " antiRo(Frayne)0.2mg (" ")
" 7: " antiRNP(Windsor)0.05mg(" ")
" 8: " antiRNP(Windsor)0.05mg(" ")
" 9-12: the same vol of extracted protein(1μl)
" 9: in PBS
" 10: " normal IgG(NG)0.06mg
" 11: " antiRo(Frayne)0.2mg
" 12: " antiRNP(Windsor)0.05mg

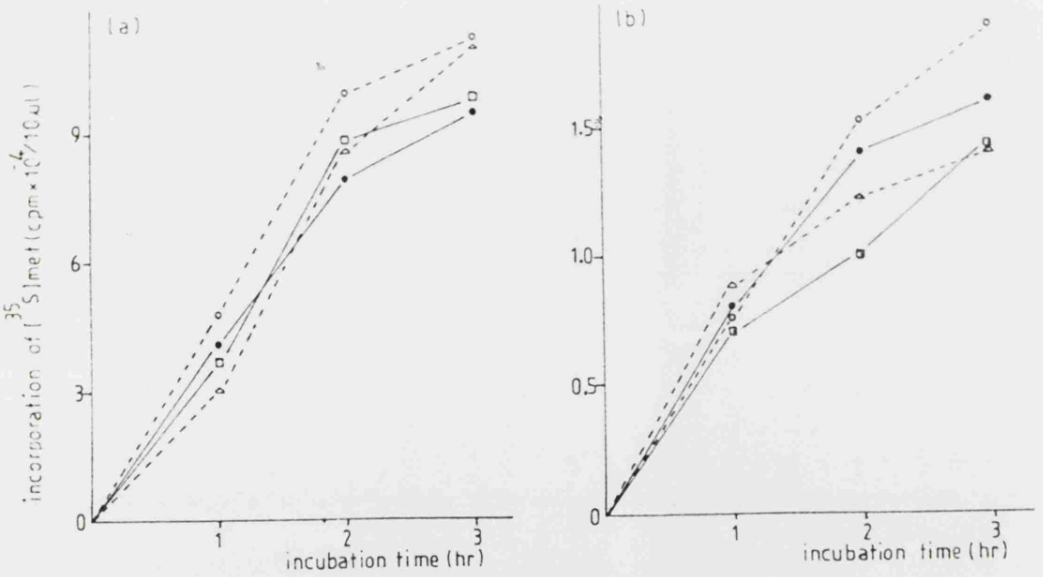
Exposure: 150,000-180,000 cpm; 1 day; -70°C.

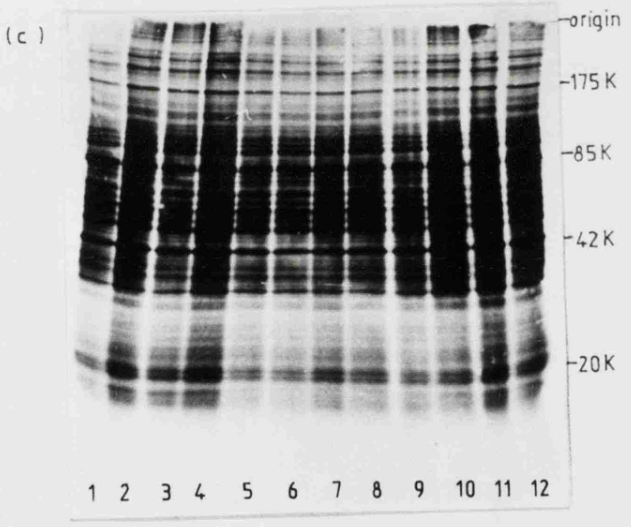
d)

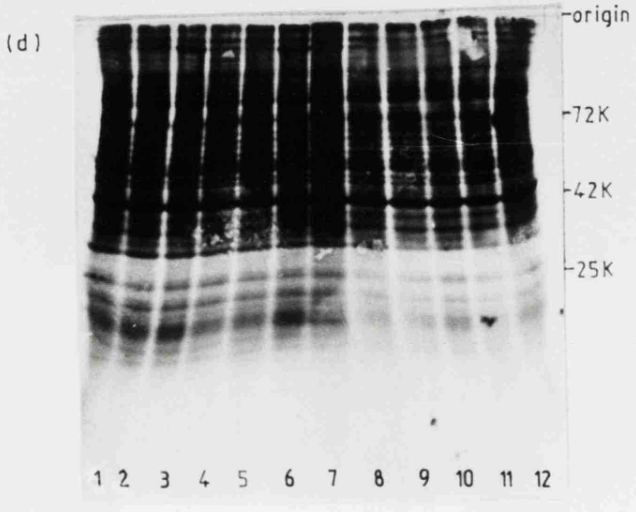
lane 1: in PBS(duplicated samples)
" 2: " PBS(" ")
" 3: " normal IgG(NG)0.06mg(duplicated samples)
" 4: " normal IgG(NG)0.06mg(" ")
" 5: " antiRo(Frayne)0.045mg(" ")
" 6: " antiRo(Frayne)0.045mg(" ")
" 7: " antiRNP(Windsor)0.05mg(" ")
" 8: " antiRNP(Windsor)0.05mg(" ")
" 9-12: the same vol of extracted protein(2μl)
" 9: in PBS
" 10: " normal IgG(NG)0.06mg
" 11: " antiRo(Frayne)0.045mg
" 12: " antiRNP(Windsor)0.05mg

Exposure: 150,000-162,000 cpm; 20 hr; -70°C.

Fig. 6.15


(a)
incorporation of [35S]met (cpm × 10⁻⁴/10 µl)
9
6
3
0
1
2
3
incubation time (hr)
(b)
1.5
1.0
0.5
0
1
2
3
incubation time (hr)


(c)
origin
175K
85K
42K
20K
1 2 3 4 5 6 7 8 9 10 11 12


(d)
origin
72K
42K
25K
1 2 3 4 5 6 7 8 9 10 11 12

Chapter Seven Discussion.

7.1. Effect of ANAs on protein synthesis in cell-free systems.

7.1.1. Significant inhibition by ANAs

7.1.1.a. Control of cell-free systems.

7.1.1.b. Type of cell-free system and mRNA.

7.1.2. Correlation of inhibitory effect to specific ANAs.

7.1.2.a. Type and amount of ANAs.

7.1.2.b. Depletion of specific ANAs.

7.1.2.c. Detection of specific antigens in lysates.

7.1.3. Mechanism of inhibitory effect.

7.2. Effect of ANAs on protein synthesis in intact cells.

7.2.1. Efficiency of transfer of IgG into intact cells.

7.2.2. Effect of ANAs on protein synthesis in cells.

7.2.3. Summary

7.3. Correlation between inhibitory effect on protein synthesis by ANAs and pathological mechanism of SLE disease.

7. Discussion.

7.1. Effect of ANAs on protein synthesis in cell-free systems.

7.1.1. Significant inhibition by ANAs.

The optimum conditions for translation of each message (TMV RNA, globin mRNA, poly(A)$^+$RNA from K562 and NS 1 cells, and poly u) in cell-free systems, either wheat germ lysate or rabbit reticulocyte lysate, have been determined and are described in chapter 4. High efficiency of protein synthesis was shown in these cell-free systems. Its efficiency was dependent on many factors, such as incubation time, mRNA concentration, amount of lysate, K^+ and Mg^{2+} salt concentration and in wheat germ lysate, spermidine concentration.

The optimum conditions for translation of these messages in cell-free systems were used in experiments to study the effect of autoantibodies from SLE patients (or antinuclear antibodies, ANAs) on protein synthesis. IgG fractions at various concentrations, from either normal or SLE patients, were added to the reaction mixture, which resulted in a change in the amount of lysate, protein (added IgG) and salt concentration from the optimum condition described in chapter 4. The sensitivity of protein synthesis to the inhibition effect by ANAswas determined and it was found that some ANA samples had an inhibitory effect on protein synthesis in these cell-free systems.

7.1.1.a. Control of cell-free systems.

Since the cell-free protein synthesizing system was sensitive to many factors, these systems were controlled to confirm that the inhibitory effect on protein synthesis resulted from ANA samples.

In each experiment, the control was normal IgG which was added to the reaction mixture at the same concentration as ANAs There was no effect on protein synthesis at concentrations of normal IgG as high as 0.4µg/µl when the incorporation of radioactivity was compared to reaction mixture with added distilled water or PBS. In contrast, a slight stimulatory effect on protein synthesis was found when using these normal IgG samples. The average stimulation by normal IgG at a concentration of 0.2µg/µl for 60 min of incubation was 3.21 $\pm$ 11.03 (n=10) for TMV RNA translation in wheat germ lysate and 2.98$\pm$11.94(n=10),3.77$\pm$13.14(n=9),and 0.75$\pm$6.59(n=5),for translation of TMV RNA,globin mRNA and poly u in reticulocyte lysate, respectively. BSA (at a concentration of 0.2µg/µl) and calf thymus DNA(at a concentration of 0.003 A_{260} units/µl) also showed no effect on these cell-free protein synthesizing systems (Fig. 5.4.b. lane 3, 4 and 6). The inhibitory effect on protein synthesis by ANAs was not due to the amount of added protein or a change in the amount of lysate.

Another factor was salt concentration. High efficiency of proteins synthesis was achieved when the reaction mixture in cell-free systems contained optimum concentrations of K^+ salt, Mg^{2+} salt and spermidine, as presented in chapter 4. At higher or lower concentrations of these salts, the incorporation of radioactivity was significantly reduced. This result was also reported by Suzuki 1977 and 1981, Ganoza et al. 1982, and Bhargava 1983. Weber et al (1977) showed that at high concentrations of Cl^-(>80mM) in cell-free systems, the binding of mRNA to ribosomes to form the initiation complex was severely inhibited. The data in chapter 4 demonstrated that different mRNAs had different optimum salt requirements for translation. Similar effects were shown with both

Mg(OAc) and KCl concentrations, which gave optimum concentrations for translation of different mRNAspecies (Suzuki, 1977; Alonoso et al., 1979; Bhargava, 1983). Other ions, such as Cd^{2+}, Ag^{+}, Cu^{2+}, Pb^{2+}, NaF, $NaHSO_4$, and $Na_2S_2O_5$ also inhibited the activity of eIF-2 phosphoprotein phosphatase, which is associated with the phosphorylation of the 38K subunit of initiation factor, eIF-2 (Ranu and Bhala, 1981). Moreover, it was reported that the inhibition of globin synthesis in rabbit reticulocyte lysate by $m^7G^{5'}p$ (Weber et al., 1978), by dsRNA (Banglioni et al., 1978) and by poly (dT) (Suzuki et al., 1980) depended on the concentration of K^{+} salt. Greater inhibition of globin synthesis by poly (dT) was observed with a higher concentration of K^{+} salt (either KCl or K(OAc)) and the inhibition appeared to be at the initiation step, but the mechanism was unknown (Suzuki et al., 1980). At certain concentrations, many salt ions showed inhibitory effects on protein synthesis in cell-free systems. In the study of the effect of ANAs on protein synthesis, the IgG fractions from normals and SLE patients were in PBS (at concentration of 0.15M) which contained NaCl, KCl, Na_2HPO_4 and KH_2PO_4 and which was diluted with H_2O to the required concentration before adding into the reaction mixture. Therefore, the amount of salt in these IgG samples was determined and quoted as the conductivity value (summarized in Table 5.3). The effect of PBS, at various concentrations, on translation of TMV RNA in both cell-free systems was studied and it was found that the greater inhibition of protein synthesis was observed with a higher amount of salt (PBS) (Fig.5.4.a.and 5.14). The wheat germ lysate cell-free system was more sensitive to salt concentration than rabbit reticulocyte lysate cell-free systems. At a conductivity of about 9μmho, the inhibition of TMV protein synthesis by PBS was approximately 5% and 15%, respectively, in reticulocyte lysate and wheat germ lysate. Many factors including RNase activity were responsible for the low

efficiency of wheat germ lysate for synthesis of large polypeptides, as previously described in chapter 4. The conductivity of ANA samples and normal IgG was between 2.5 and 9μmho. Therefore, the inhibitory effect by ANAs was not due to salt concentration, as shown in Fig.5.4.a.and 5.14, which compares conductivity to percentage inhibition of TMV protein synthesis in both cell-free system by these ANAs and normal IgG samples. Phosphate buffer at a high concentration (3.63mM and 9.09mM) inhibited TMV protein synthesis in rabbit reticulocyte lysate (Fig. 5.4 b. lane 11 and 12) and there was no effect at concentration of 1.81mM. PBS solution in the reaction mixture contained only 1.44mM Na_2HPO_4 and 0.36mM KH_2PO_4, so that it should not show any inhibitory effect on protein synthesis.

The inhibitory effect on TMV or globin protein synthesis in both cell-free systems was found to depend only on the amount of added ANAs. The ANA and normal IgG samples were diluted with PBS so that the conductivity was unchanged whereas the amount of IgG was varied. For example antiRo at concentrations of IgG between 0.025-0.2μg/μl had conductivity values of 11.5-13 μmho. The incorporation of radioactivity in controls (added normal IgG) was nearly the same at various IgG concentrations whereas decreased incorporation of radioactivity was observed with increasing the amounts of added ANAs(Fig.5.5.a,5.10.a and 5.15.a). These results clearly showed that the inhibitory effect on protein synthesis in cell-free systems was from specific ANAs and that it was dose-dependent.

7.1.1.b. Type of cell-free system and mRNA.

To test whether these ANA samples can function as general translational inhibitors, their effects on translation of mRNA from

various sources were examined in cell-free extracts derived from rabbit reticulocyte and wheat germ.

TMV RNA was translated in either reticulocyte lysate or wheat germ lysate and in the presence of ANAs. The results showed that TMV protein synthesis was efficiently inhibited by certain ANA samples in both cell-free systems (Table 5.2 and 5.6). The extent of inhibition, however, varied with the systems studied, for example with antiLa (Buffalo)at a concentration of 0.2μg/μl, TMV protein synthesis was inhibited by 36.6% and 49.0%, respectively, in rabbit reticulocyte lysate and wheat germ lysate, over 60 min of incubation. Some ANA samples, antiSm/Ro (Pryce), antiRNP (Johanna) and antiLa (Knowland), showed no inhibition of protein synthesis in reticulocyte lysate systems but gave more than 30% inhibition when added to wheat germ lysate systems. However, some of ANA samples gave less effect in wheat germ lysate systems, for example, antiRo (Frayne) and antiDNA(Jonas). Protein synthesis in wheat germ lysate seems to be affected by ANAs more than in reticulocyte lysate. This result was observed in the inhibitory effect of cibacron blue F36A (dye) on polypeptide synthesis (Wu,1980). He found that addition of 13.4μM and 110μM dye into wheat germ lysate and reticulocyte lysate, resulted in a 66% and 68% inhibition of protein synthesis, respectively. In addition, these two cell-free systems had different efficiency for optimal synthesis of a particular protein (Scheele and Blackburn, 1979). This higher inhibition of protein synthesis in wheat germ lysate seems to relate to its sensitivity to salt concentration as described in a previous section (7.1.1.a.). The maximum inhibition of protein synthesis in wheat germ lysate was approximately 80% (Fig.5.14), at high concentration of PBS solution, while this value was in-

creased to 90% inhibition in rabbit reticulocyte lysate (Fig.5.4.a).

Rabbit reticulocyte lysate appeared to offer a highly suitable cell-free synthesizing system for testing the effect of ANA samples. In order to gain more information on the nature of inhibitory effects by these ANA samples, the effect of ANAs was studied in translation of various types of message , such as TMVRNA, globin mRNA, poly(A)$^+$RNA from K562 and NS 1 cells and poly u, in reticulocyte lysate cell-free systems. The inhibition of synthesis of these proteins by antiRo (Frayne) and antiLa (Buffalo) was different as presented in Table 5.2, 5.4, and 5.5 and section 5.2.3.b. However, antiRo (Frayne) which gave a high inhibitory effect still showed a high effect in translation of all types of message but the inhibition was lower with poly(A)$^+$RNA from K562 and NS 1 cells and poly u. Therefore,a similar factor was presumably involved in this ANA effect. On the other hand, the inhibitory effect by ANAswas limited by factors which were needed for translation of all these RNAs. Bathurst et al. (1980) and Bathurst and Smith (1982) also demonstrated that the presence of the nuclear poly(A)$^+$RNA from mammary glands totally inhibited the synthesis of all proteins directed by several exogenous mRNAs such as reovirus protein, milk protein, liver protein and rabbit globin protein in both reticulocyte lysate and wheat germ lysate cell-free systems. Thus the inhibition did not appear to be mRNA-specific. They concluded that the inhibitory RNA species seem to be a general translational inhibitor since neither the nature of the cell-free system (plant or animal-derived) nor the source of the mRNA was important.

7.1.2. Correlation of inhibitory effect to specific ANAs.

7.1.2.a. Type and amount of ANAs.

The antibodies in sera of patients with connective tissue diseases such as SLE are specifically directed against nuclear and cytoplasmic antigens (as described in introduction section 1.7.2.) Some of these antigens (small ribonucleoprotein (RNP) particles) are complexes of low M.W. RNAs and proteins and are found in all eukaryotic cells. Little is known about the precise cellular function of these small RNP particles, although there was a suggestion that snRNPs participate in the splicing of pre mRNA (Lerner et al., 1980; Roger and Wall, 1980; Mount et al., 1983). Current evidence suggests that most mRNA precursors are spliced by a common mechanism and the splicing reaction requires snRNA (U1 RNA)(Yang et al.,1981; Hernandez and Keller, 1983; Padgett et al., 1983a and 1983b). The actual mechanism of RNA splicing is still unclear. To examine the role of these small RNP particles in the living cell, including their relationship to the onset of autoimmune disease , the study of the nature of these small RNPs particles and the effect of their antibodies on transcription and translation process is of interest.

Serum samples (n=38) from SLE patients were divided into 8 groups by the specificities of their autoantibodies and some disease symptoms (Table 5.1.). The IgG fraction was prepared and tested for specificities using immunoprecipitation, radio-immunoassay and ELISA techniques including determination of protein concentration (Table 5.1.). IgG samples from both normals and SLE patients (at the concentration of 0.2µg/µl)were added to a cell-free mRNA translation system from rabbit reticulocyte lysate or wheat germ lysate.

It has been shown here that these ANAs seem to have a sig-

nificant effect on proteins synthesis in cell-free systems. Some of ANA samples can specifically inhibit in vitro translation of various mRNAs. The inhibitory effect was found to be dependent on the amount of IgG added (Fig.5.1.a). Since no effect was observed using normal human IgG, the inhibitory effect of ANAs could not be attributed to a non-specific effect of IgG. The data also indicated that the quantitative effect on protein synthesis by ANAs was very significant, which was shown from the reduction in the incorporation of radioactivity and the protein pattern when analyzed by SDS-PAGE and fluorography. All types of proteins were synthesized in reduced amount in samples which gave an inhibitory effect and no protein was synthesized at high percentage inhibition (Fig.5.2, 5.9, and 5.13). The TMV protein at M.W. of 160K was not found in translation products from wheat germ lysate. This protein is a readthrough product of protein with M.W. of 110K and it is not a major product (Pelham,1978) in TMV RNA translation in cell-free systems. Therefore, this TMV protein (160K) seems to be affected by ANAs before other types of protein and was not found in translation products at high percentage inhibition (higher than 30%) (Fig.5.2.a. lane 5 and Fig. 5.2.b. lane 3 and 7). This result might be related to the low amount of this protein which is synthesized in cell-free systems. From the results, it could be concluded that these ANAs did not act on the translation of specific mRNA species, since their inhibitory effects gave nearly total inhibition of protein synthesis for many types of proteins.

The inhibitory effect on protein synthesis in both cell-free systems was shown to be independent of the particular types of ANAs as shown in Fig.5.3, 5.8, and 5.12, for the comparison

of these values. ANA samples from different groups inhibit cell-free protein synthesizing systems, though to different degrees. One sample each from the antiRo and antiDNA groups gave very high inhibition (about 95%) and both of them showed the highest positive value when testing for their antibody specificities by ELISA (Table 5.1). The same result was given for antiLa (Buffalo). Other ANA samples in these groups (antiRo, antiDNA and antiLa) showed less effect on protein synthesis. The inhibition was compared with the amount of added IgG, not with the amount of specific autoantibodies. Therefore, each of these ANA samples may have different amounts of specific autoantibodies and this may result in different degrees of effect on protein synthesis. This is one possibility, but the results are not conclusive and some ANA samples which gave low ELISA values, gave a high percentage inhibition of protein synthesis (antiRo (Cuff) and antiDNA (Holman)). Thus it is possible that inhibitory activity may not be related to the ANA specificity identified. Most samples from antiSm and antiRo/La groups gave low inhibition (less than 30%) for both TMV RNA and globin mRNA translation in rabbit reticulocyte lysate system, whereas most antiRNP samples had no effect on the synthesis of these proteins (less than 10% inhibition).

My observations for antiRNP group on protein synthesis in cell-free system were not entirely consistent with the results of an inhibitory effect in cell-free protein synthesis by these snRNA. Isolated U1 snRNA (Rao et al., 1977.a) low M.W. RNA (5.5.S) from nuclear RNP particles of adenovirus-infected HeLa cells (Sarma et al.,1978), poly(A)$^+$ small nuclear RNA from mammary glands (Bathurst et al., 1980, Bathurst and Smith, 1982) and low M.W. RNA from rat liver free cytoplasmic mRNP particles (Kühn et al., 1982) have been

shown to inhibit cell-free protein synthesis directed by various mRNAs. By kinetic studies of the mechanisms responsible for the inhibitory effect, it was demonstrated that some, such as low M.W. RNA (5.5.S) and poly(A)$^+$snRNA (species F), inhibited at the level of initiation of protein synthesis (Sarma et al.,1978; Bathurst and Smith, 1982) whereas others appeared to prevent elongation (Rao et al.,1977.a;Bathurst et al., 1980; Bathurst and Smith, 1982). Anti U1-RNP antibodies did not prevent the inhibition of protein synthesis observed in the HeLa cells during mitosis (Smith et al.,1983).

My results on the inhibitory effect of antiRNP antibodies on cell-free protein synthesis cannot be directly related to the proposed mechanism of mRNA splicing which required U1 RNA. Lenk et al, (1982) have shown that transfusing antiRNP antibodies into infected cells with liposomes selectively inhibits viral fiber and hexon synthesis in adenovirus infected cells. Addition of specific anti-sera from SLE patient (against U1 RNA) into isolated nuclei (Yang et al., 1981), in a coupled transcription-processing system (Padgett et al., 1983a) and in nuclear extracts or whole cell extracts of HeLa cells (Hernandez and Keller,1983; Padgett et al., 1983 b) resulted in specifically inhibited splicing of adenovirus pre-mRNA. These results suggested that U1 RNP is essential for the splicing of mRNA precursor. There are several possible explanations for differences between these results. One possibility is related to the system used, since they studied the adenovirus RNA splicing and translation in intact cells or cell and nuclear extracts, and also there are possible differences in antiRNP antibodies,which may be directed against a different class of particles with different specificity. More data is needed to give a complete explanation.

7.1.2.b. Depletion of specific ANAs.

To ascertain the specificity of inhibitory effect of some ANA samples on cell-free protein synthesis, the antisera (antiRo (Frayne) or antiLa (Buffalo)) were adsorbed with antigen Ro and antigen La covalently linked to insoluble matrixes (Sepharose 4B) and specific IgG were prepared from the eluates. For the antiRo (Frayne) sample after passing through the antigen Ro-Sepharose 4B column, the specificity of antiRo in both bound and unbound fractions was lost (Fig.5.7.a.) Depleted IgG was obtained, but not specific IgG. These depleted IgGs lost their capacity to inhibit TMV RNA translation in reticulocyte lysate systems (section 5.2.2.f.).

For antiLa (Buffalo), the bound fraction, which contained mostly specific antiLa, was found to show a higher inhibitory effect (about 5 fold) than the IgG sample before passing through the column or the unbound fraction (section 5.2.2.f.). In the preparation of the La-Sepharose column, the percent coupling was only 16.5% which may be due to using PBS during the coupling stage (March et al., 1974). Therefore, this resulted in an unbound fraction which still had residual antiLa activity.

These results seem to show that inhibitory effects on protein synthesis in cell-free systemswere related to the activity of specific antibodies. However, the unbound fraction of antiLa, which had nearly the same activity as the bound fraction (by ELISA test, Fig.5.7.b), showed very low inhibition (less than 10%). Therefore, the specificity of the inhibitory effect by ANAs is still unclear.

7.1.2.c. Detection of specific antigens in lysates.

The ANA samples which were used in these experiments were characterized by detecting their antigenic polypeptide targets using the immunoblotting technique. Crude and purified antigen from calf thymus extract and human spleen extract and cell-free translation lysates (reticulocyte lysate and wheat germ lysate) were used as a source of antigen. The antigens detected by antiLa-, antiRo-, antiSm- and antiRNP-antibodies were identified and are summarized in Table 5.7 and Fig.5.16. Different sized antigens were detected using antisera of the same specificity from different patients. This result was also found by Wooley et al, (1983). This may result from the sensitivities of the technique used or from artefacts. Alternatively, the sera from these patients may detect a different class of antigenic polypeptides. The source of antigen or degradation and aggregation of antigens during their preparation can also cause variation in the determination of these antigenic polypeptides (see MacGillivray et al, 1982 for review).

Using antiLa and antiRo samples, the major proteins associated with these antigens are 3 components with M.W. of 52K, 45K, and 31K and 2 components with M.W. of 63K, and 54K, respectively, (Table 5.7). This result is similar to Steitz et al. (1982) who showed that antiLa-and antiRo-antibodies recognized protein at M.W. about 50K. The lower M.W. antigenic polypeptides of La antigen might be degradation products of the 50K polypeptide (Wooley et al, 1983).

AntiRNP samples recognized 6 polypeptide bands, one at 104K, two at 60-65K and three or four at 30K, whereas antiSm samples recognized 3 polypeptide bands, one at 120K and two at

60-65K (Table 5.7). These results were different from other investigators, since these antiSm-and antiRNP- .sera did not identify proteins at M.W. around 13K (Steitz et al., 1982; White et al., 1982; MacGillivray et al., 1982; Wooley et al., 1983; Takano et al., 1981). Wooley et al. (1983) suggested that the smaller polypeptides recognized by antiRNP antisera were degradation products of 68K polypeptides. My results also showed antigenic protein at M.W. approximately 65K for both antiRNP and antiSm. These discrepancies might result from different sources of antigen or from degradation of antigen.

Only antiLa-and antiRNP-sera could identify the protein at M.W. approximately 50K (antiLa) and 55K and 30K (for antiRNP) in rabbit reticulocyte lysate. Either wheat germ lysate or rabbit reticulocyte lysate examined for antigenic protein with antiRo antibodies. These antiRo antibodies failed to recognize any proteins in these lysates. These results show that the lysates did not contain any Ro antigen, within the limits of sensitivity of the immunoblotting technique. More data is needed to give definite conclusions on the relationship between antibody specificity and inhibitory effects on protein synthesis in cell-free systems.

7.1.3. Mechanism of inhibitory effect.

The mechanisms responsible for the inhibitory effect of ANAs on TMV RNA and globin mRNA translation in rabbit reticulocyte lysate were examined. To investigate whether ANA-dependent inhibition occurred at the level of chain initiation or elongation of protein synthesis,the kinetics of inhibition were compared in a time-course experiment with those of inhibition by aurin tricarboxylic acid and cycloheximide, known inhibitors of initiation

and elongation, respectively. The experiment showed results the same as Bathurst et al. (1980) and Bathurst and Smith (1982).

Mathews et al. (1971) and Fresno et al. (1976) have shown that aurin tricarboxylic acid blocked initiation in the reticulocyte system by interfering with the binding of mRNA to the 40S-ribosomal subunit. Mathews (1971) demonstrated that aurin tricarb oxylic acid preferentially inhibited chain initiation on messenger lacking the specific initiation codon (AUG and GUG), such as poly u and higher concentrations of aurin tricarboxylic acid were required to interfere with initiation on natural messengers. My result was similar in that aurin tricarboxylic acid showed higher inhibition of poly u translation than TMV RNA or globin mRNA translation in reticulocyte lysate systems(section 5.2.3.c., Fig. 5.6 a.and b. and Fig 5.11.a). Aurin tricarboxylic acid specifically blocked the formation of the ternary complex (Met-$tRNA_f$, IF-E_2,GTP) (Fresno et al., 1976). Lodish et al. (1971) also showed that the blocking at initiation of haemoglobin synthesis in reticulocyte by aurin tricarboxylic acid was dose-dependent and at higher concentration it also blocked some stages in the elongation of nascent polypeptides (Lodish et al., 1971). The primary effect of cycloheximide was quite different and was to inhibit translocation of the ribosome along the mRNA (Lodish et al., 1971; Vázquez, 1974). It can also block initiation at a higher concentration than needed to block elongation (Lodish et al, 1971). My results (Fig.5.6 a. and b. and Fig.5.11.a.) indicate that inhibition by antiRo (Frayne) and antiDNA (Jonas) occurred predominantly at the level of chain elongation, since the kinetic curves mimicked cycloheximide. AntiLa (Buffalo) and antiRo (Cuff) behave like aurin tricarboxylic acid which inhibited at initiation.

The other ANA samples, antiSm/RNP (Summes), antiDNA (Hildea A) and antiRo/La (Jone) were also studied in a time-course experiment of TMV RNA translation in reticulocyte lysate. They were similar to aurin tricarboxylic acid except that the incorporation of radioactivity was slightly higher (Fig.5.6.b).

This result was clearly shown after analysis of their translation products on SDS-PAGE and fluorography (Fig.5.6.c. and 5.6.d. and Fig 5.11.b). The accumulation of incomplete translation products(protein with M.W. less than 70K) was shown in the cell-free system which had inhibition at the elongation step. This result is supported by the observation of Asselberg et al.(1980). A lower amount of low M.W. protein followed by accumulation of completed protein was seen in translation products of samples which showed inhibition at initiation.

By studying poly u translation in the presence of some ANA samples in the reticulocyte lysate system, a result was obtained which confirmed the mechanism for the inhibitory effect of ANAs. Since antiLa (Buffalo), antiRo (N.Cuff) and antiSm/RNP (P.Summes) slightly affected translation of poly u (Table 5.5.) they might interact with initiation factor. AntiRo (Frayne) and antiDNA (D.Jonas) still gave quite high inhibition (Table 5.5). It was likely that they interfered at the elongation step.

7.2. Effect of ANAs on protein synthesis in intact cells.

Some ANA samples showed significant inhibitory effects on protein synthesis in cell-free systems, as previously discussed in section 7.1. Since Alarcon-Segovia et al.(1978) have demonstrated that antiRNP antibodies can penetrate into viable cells through Fcγ receptors, and Lenk et al.(1982) have demonstrated

inhibition of adenovirus protein synthesis in infected HeLa cells by antiRNP antibodies transferred into cell using liposome, this work was extended to the in vivo situation (the intact cells) to test for a pathological role of ANAs in intact cells.

ANA samples that showed high inhibition in cell-free systems (antiRo antibodies (Frayne)), including antiRNP antibodies, were tested for their effects on protein synthesis in intact cells. Cells were incubated with ANAs or normal IgG, which was introduced into cells by various techniques (to be discussed in next section 7.2.1.), before pulse-labelling protein with ^{35}S-Met. The effect of ANAs was analyzed by comparing the amount of radioactivity incorporation into protein and the gel pattern of the synthesized protein with control cells which were treated with PBS or normal IgG.

7.2.1. Efficiency of transfer of IgG into intact cells.

The techniques used for introducing IgG molecules (normal IgG and ANAs) into cells were liposomes or red cell ghosts mediated transfer, use of cell lines with a high percentage Fcγ receptor and permeabilization procedures. The efficiency of these techniques (both qualitative and quantitative) was checked using the labelled material, ^{125}I-IgG or FITC-IgG.

Liposomes have recently been used to introduce a variety of biological molecules and molecular complexes into cells (as described in Introduction section 1.7.3.b.) The liposomes used in this experiment were reverse phase vesicles which were reported to be the most effective liposome to encapsulate an aqueous phase (Szoka and Papahadjopoulos, 1978). A high degree of aqueous phase encapsulation, about 19-22% for cytochrome c (the same as reported by Lenk et al., 1983) and 30-36% for ^{125}I-IgG, was found (Table

6.2 and 6.3). This value was only 13% when using multilamellar vesicles to encapsulate ^{125}I-IgG and less than 1% of total radioactivity was attached to cells (Magee and Miller, 1972). My result gives slightly higher amounts of ^{125}I-IgG attached to cells, about 1-2% of total radioactivity, after subtracting the amount of radioactivity in the control (Table6.3).Fluorescent microscopy of RPMI 1788 cells incubated with FITC-IgG containing liposomes (Fig.6.7) showed that some of these cells had fluorescence distributed throughout all the cell but most cells had fluorescence around the surface. Weinstein et al. (1977) suggested a generalized distribution of fluorescence in cells (6-carboxylfluorescein) indicating releasing of entrapped material from liposomes into the cytoplasmic space. Ryman and Tyrrell (1980) suggested that the recovery of liposomes in association with cells did not necessarily mean that its content had been incorporated into the cells. From these results, liposomes can introduce IgG into cells, but the efficiency is rather low. The rate-limiting step in the transfer of macromolecules using liposomes is liposome-cell interaction. This interaction is a complex phenomenon and many possible mechanisms have been proposed (see Poste, 1980;and Ryman and Tyrrell, 1980 for review). Many factors, properties of the liposome membrane, the cell type and environmental factor (temperature) are involved in the liposome-cell interaction (Poste, 1980). An important factor in this interaction is the fluidity of the liposome (Pagano and Weinstein, 1978). However, fluidity of the liposomes also results in the loss of encapsulated material during incubation with cells at 37^{o}C (Weissman et al., 1977). Szoka et al.(1979) suggested that adsorption of liposomes to cell surfaces might increase leakage of encapsulated material. The presence of cholesterol

in the liposome membrane reduced the permeability of phosphatidyl choline vesicles induced by various proteins (Papahadjopoulos et al., 1973). The binding of liposomes to cell surface protein might promote permeability changes and result in transfer of contents into the cells (Pagano and Takeichi., 1977). The liposomes used in this experiment were composed of phosphatidyl choline and cholesterol. Therefore, the low percentage binding of liposomes to cells might be related to the presence of cholesterol in the vesicle surface. However, several non-exclusive mechanisms and many factors result in the partial loss of liposome contents and low efficiency in liposome-cell interactions (Ryman and Tyrrell, 1980). These mechanisms are not clearly understood.

The liposome could be applied to cells at high concentration without significantly affecting cell function (Fig.6.6) and cell viability (section 6.2.2.a. iii). This result was also shown by Weinstein et al. (1977) and Wilson et al. (1979). Many reports document the use of loaded red cells (or red cell ghosts) for the microinjection of macromolecules into cultured cells. This method has been shown to be a simple and efficient way to transfer significant amounts of proteins and nucleic acids (in introduction section 1.7.3.a). In my experiments, the pre-swell technique has been used to load red blood cells with macromolecules (IgG) and the loaded red cells were then fused to cultured cells (X 63 mouse cells and K562 cells) using polyethylene glycol (PEG) (the results were in section 6.2.2.b). The overall efficiency of red cell-mediated microinjection under these conditions was tested using ^{125}I-IgG and it was found that about 20% of radioactivity was entrapped in the red cells and only 0.06% as transferred to target cells. The uptake by red cells is prop-

ortional to the external concentration of protein but does not relate to the change of the molecules. Thus, both protein and nucleic acid can be loaded (Rechsteiner, 1975; Schlegel and Rechsteiner, 1978). The pre-swell technique offered greater entrapment of exogenous macromolecules and it was found that the uptake of ^{125}I-IgG, ^{125}I-IgG-myoglobin and protamine mRNA was 42-60%, 36-40%, and 12-15%, respectively (Rechsteiner, 1975; Boogaard and Dixon, 1983 a). In the pre-swell technique, exogenous molecules were introduced into red cell without prior removal of red cell contents,therefore protein or nucleic acid which is sensitive to oxidation might be denatured (Schlegel and Rechsteiner, 1978; Boogaard and Dixon, 1983 a). This result can be disregarded, since Antman and Livingston (1980) and Smith et al. (1983) found that biologically active IgG (either IgG against SV 40 antigen or anti U1 RNP antibodies)could be detected after introduction into the cell by this technique.

Transfer of the entrapped IgG into cells was found to be approximately 0.06%, which was higher to what has been reported before, (about 0.02% of IgG (Wasserman et al., 1976 and Smith et al, 1983) and only 0.005% of protamine messenger (Boogaard and Dixon, 1983 a and 1983 b)). Polyethylene glycol, which was normally used to inject large number of cells,showed limitations in the number and type of cells that can be treated, fusion efficiency which was generally low,and lack of selectivity (Furusawa, 1980). Godfrey et al. (1983) reported that fusion efficiencies varied with the length of contact between cells and fusion medium as well as with PEG concentration. Longer exposure up to 2 min, somewhat enhanced fusion but greatly reduced viability. My results showed that about 12-23% of cells (K562 and X 63) died during

fusion which was the same (approximately 20%) as reported by Boogaard and Dixon (1983 b). These results indicated that the microinjection procedure using red cell ghosts as protein carriers, can introduce IgG into cells but does not allow the transfer of a massive amount of protein, as also shown by Boogaard and Dixon (1983 a and 1983 b) and Smith et al. (1983).

It has been shown that antiRNP-antibodies can penetrate viable human mononuclear cells (MNC) via their Fcγ receptor (Alarcon-Segovia et al., 1978). They demonstrated the presence of nuclear, cytoplasmic, and membrane fluorescence in MNC after incubation of these cells with high titre FITC-antiRNP antibodies at high protein concentration (42mg/ml). They also showed that longer incubation (more than 1 hr) did not increase the number of viable cells with intranuclear speckled fluorescence. Based on this result, attempts were made to introduce ANA samples including normal human IgG into viable cells, through their Fcγ receptors by incubation for 2 and 19 hr. The cells were then checked for effects on protein synthesis. K562 cells, which have a high percentage of Fcγ receptor detected by rosette assay (approximately 67%), were used in this experiment. The mean value of percentage Fc receptors for K562 cells was lower than reported by Klein et al. (1976) (90-95%), which may result from the conditions and properties of antibody coated sheep erythrocytes used.

The high concentration of homogeneous antibodies of predominantly IgG class may promote their penetration into viable cells (Alarcon-Segovia et al., 1978). In my experiments, the highest concentration of ANAs was 20mg/ml (antiRo, Frayne) therefore, the penetration of ANAsinto cells may not be as efficient as reported by Alarcon-Segovia et al. (1978). Neither ^{125}I-IgG nor FITC-IgG were used to test the efficiency of this procedure

because the concentration of protein was only 0.15 mg/ml and 8.4 mg/ml, respectively. After incubation of K562 cells with ANAs or normal IgG, the viability of the cells was beween 85-96% (Table 6.7), showing no affecting by ANAs as reported by Alarcon-Segovia et al. (1978).

Attempts to introduce a large amount of ANAs into cells were not successful thus far. I decided to use the permeabilization procedure since Halegoua et al. (1976) and Bergan (1978) had reported that the technique was simple and the cells were freely permeable to macromolecules (phosphorylated component and exogenous protein) and efficiently synthesized DNA, RNA, poly (adenosine diphosphoribose) and membrane proteins. My permeabilization procedure was different. The cells were permeabilized in hypotonic buffer (without toluene, sucrose and mercaptoethanol) which contained ANAs or normal IgG for a short time before adding a hypertonic buffer. The recovery of the cells was 56-70% and the viability was reduced to 75-85% (section 6.2.2.d). These permeable cells were functionally normally, giving the same incorporation of radioactivity and a similar gel pattern as controls. IgG-containing cells were obtained by this procedure as shown by using ^{125}I-IgG and FITC-IgG. About 0.5-0.7% of total radioactivity was found in the permeable cells whereas this value was only 0.16-0.3% in controls (adding ^{125}I-IgG after the cells were resealed). The percentage of fluorescent cells was more than 70% in the sample and less than 20% in the control. The value in the control correlated with the amount of dead cells after permeabilization (15-25%). These results indicated the entry of ANAs into cells although at a low concentration.

7.2.2. Effect of ANAs on protein synthesis in cells.

Analysis of the protein product of cells was carried out by radiolabelling, extracting or immunoprecipitating the protein and analyzing by gel electrophoresis. After labelling cell protein with ^{35}S-Met, the cells were extracted with buffer containing detergent (NP40) which effectively solubilized membrane proteins without destroying their antigenicity. Two procedures were used for analyzing the radiolabelled protein, single-dimensional and two-dimensional SDS-PAGE. The protein bands and spots were visualized by fluorography.

Many human cell lines, either lymphoblastoid cells(RPMI 1788),myeloma cells(RPMI 8226), chronic myelogenous leukemia cells (K562) or human plasma cells leukemia (HMy2) were used to test for optimal conditions in radiolabelling protein in intact cells, since the rate of protein synthesis in these cells was generally higher than resting cells. The data demonstrated the high efficiency of these techniques, the cells could be labelled for 3-4 hr and incorporation of ^{35}S-Met into protein was linear during this period (Fig. 6.1.) and dependent on the amount of radioactive amino acid added (Fig.6.2). The amount of ^{35}S-Met incorporated into protein generally ranged from 3-5 cpm per cell (Table 6.1). These results were similar to that reported by Mishell and Shiigi (1980).

On gel analysis, RMPI 1788 cells were shown to synthesize and secrete Ig M (λ) together with other protein components which had a M.W. range between 210K and 18K (Fig.6.3.a. lane 1-5 and Fig 6.3.b. lane 1-4). The RPMI 1788 cells in vivo synthesized and secreted μ chain and λ chain which had a mobility on reducing gel corresponding to 72K and 25K, respectively. Immunoprecipitation confirmed IgM (λ) production by RPMI 1788 cells(p.187). The M.W. of

μ chain was slightly lower than that reported by Dolby et al. (1980). They found that the secreted μ chain in vivo had a M.W. about 77K, higher than the in vitro directed product which migrated as 69K. Molgaard et al. (1981) demonstrated slight differences in the M.W. value; the mRNAs that were isolated from RPMI 1788, coded for a major μ chain (65K), a minor μ chain (67K) and a k chain (28.6K) in rabbit reticulocyte lysate systems.

Light chain (λ) was found in the protein product of RPMI 8226 (Fig.6.3.a. lane 6-9). Matsuoka et al. (1967) reported that this cell line produced λ type light chain of human immunoglobin in free form as shown by using immunodiffusion and immunoelectrophoresis. HMy2 cells also synthesized and secreted IgG (k) as detected by gel electrophoresis of protein products(Fig.6.3.b lane 6-9) and immunoprecipitation using rabbit anti human-IgG (k) (p.187). The $IgG_{(k)}$ synthesized by HMy2 cell was the same as reported by Berk et al. (1978) and Edwards et al. (1982).

The protein products of these cell lines were also investigated on two-dimensional (2D) gel electrophoresis (O'Farrell 1975), which gave the highest resolution of complex protein mixtures. The 2D gel patterns of these cell lines were similar to each other with slight differences in some spots or areas as shown in Fig. 6.4. and 6.5. The result was a complex pattern of more than 100 spots, each of which appeared in a reproducible position with respect to its neighbours. This method gave a reproducible result. However it was rather difficult to interpret differences in spot intensities (quantitative analysis) since slight changes in the intensity probably resulted from slight variation in the number of cells or their response to the labelling. Therefore, single-dimensional SDS-PAGE which is faster and enables more samples to be processed

at one time was used for examination of the effects of ANAs on protein synthesis in intact cells.

Many techniques were used to transfer IgG into cells, different types of cell lines were used dependent on their properties. For example, K562, the Fcγ receptor bearing cells, were tested in the experiment in which IgG was introduced into cells via Fcγ receptors. The effect of ANAs was determined by comparing the amount of radioactivity incorporation into protein, and gel analysis of the synthesized protein with controls to which PBS or normal IgG were added.

Using 4 techniques to transfer ANAs into intact cells, there was no effect on protein synthesis by those ANA samples which showed an effect in cell-free systems. This observation suggests either the amount of ANAs transferred was not high enough to show the effect or the physiological mechanism of these ANAs in intact cells was different from that in cell-free systems.

The efficiency of the techniques used to transfer IgG into cells was checked and discussed in section 7.2.1. The amount of IgG transferred could be calculated from the percent binding of radioactivity (^{125}I-IgG) to cells. For the experiment using liposomes 1-2% of total added ^{125}I-IgG was bound to cells and only 0.2-0.4% was actually inside the cell (calculated from the amount of radioactivity in cell lysate). This value (amount of radioactivity inside the cell) was 0.06% and 0.35-0.4% (after subtraction of the control value) of total added ^{125}I-IgG by using red cell ghosts and permeabilization, respectively. Of the ANA samples used, antiRNP (M.Windsor) had the lowest concentration of protein (5mg/ml) whereas the highest protein concentration was 20mg/ml for antiRo (Frayne). The calculated amount of ANAs inside the

cells (2×10^6) was approximately 2-6μg, 0.2-0.6μg and 0.2-0.8μg, respectively when using liposomes, red cell ghosts and permeabilization techniques. A typical mammalian cell contains about 10^{-5}μg of RNA and 1-5% of the total cellular RNA is mRNA (Maniatis et al, 1982). Therefore, 2×10^6 cells have approximately 0.2-1.0 μg of mRNA. In cell-free systems 2.2μg of ANAs was used and showed the inhibitory effect on 0.07μg of globin mRNA. Therefore, at least 7μg of ANAs would need to be transferred into 2×10^6 cells. The concentration of ANAs was lower than used by Alarcon-Segovia et al (1978) when incubating cells in ANAs to transfer ANAs into cells via Fcγ receptor. The amount of IgG transferred could not be determined but it might not be enough since there was no effect on protein synthesis in cells, unlike previous reports using other techniques. These results showed that the amount of ANAs in cells might not be high enough to affect protein synthesis. The number of proteins which were synthesized in these cells was more than those from TMV RNA which were used in cell-free systems (Fig. 6.9, 6.12.c, 6.13.c, and 6.15.c and d). By studying the effect of cycloheximide it was found that the percentage inhibition of protein synthesis in K562 cells was dependent on the amount of cycloheximide added (Fig. 6.11.a). The inhibition did not affect any specific type of protein as shown by gel analysis (Fig. 6.11.b). At low percentage inhibition (about 19%) the intensity of these protein bands was similar to controls (Fig. 6.11.b, lane 1, 11, and 12). If the synthesis of all types of protein were affected by ANAs in intact cells, as shown in cell-free systems, slight effects may not be detected by this analysis. Shuttleworth et al. (1982) also demonstrated that changes in protein patterns of treated Namalwa cells could not be detected even when the treatment of these cells

with butyrate and 5'-bromodeoxyuridine(Brd Urd)resulted in enhancement of interferon synthesis (up to 300 fold). They suggested that butyrate and Brd Urd may have a general effect on transcriptional capacity of chromatin, the pattern of proteins expressed by the cell was not necessarily disrupted.

My result was different from Lenk et al.(1982) who found that both hexon and fiber (viral protein) synthesis were reduced in the presence of antiRNP antibodies. This may be related to the different system used, since the viral protein product was more simple than proteins synthesized by human cell lines, or the difference in antinuclear antibody samples which may not have the same specificity, since my result (in cell-free systems)showed an effect on the synthesis of all types of protein.

7.2.3. Summary.

An effect of ANAs inintact cellscannot be shown due to the limitation of the efficiency of transfer of IgG into the cells. The results for transfer of macromolecules into cells were similar to those reported by other investigators (as described in section 7.2.1). A high amount of ANAs was needed to show an inhibitory effect on protein synthesis in intact cells. The protein pattern of cell lines used also was more complicated than protein synthesis in cell-free sysytems. At the low percentage inhibition (less than 20%), differences in protein pattern and intensity from controls could not be detected (as shown by the result using cycloheximide). The complexity of the proteins synthesized by these cell lines and the limited efficiency in techniques for the transfer of macromolecules made the results with intact cells different from the cell-free sysytem. Therefore, the actual physiological mechanism

of the effect of these ANAs on protein synthesis in intact cells is not known yet. It can be suggested that the inhibitory effect on protein synthesis may be shown if a high intracellular concentration of ANAscould be achieved, as in the cell-free system.

7.3. Correlation between inhibitory effect on protein synthesis by ANAs and pathological mechanism of SLE disease.

SLE is an autoimmune disease of unknown etiology. The mechanisms leading to the development of autoantibodies are not well understood. However, the appearance of specific antibodies in patient sera has been reported to be associated with particular clinical signs of disease (Notman et al., 1975). For example, the presence of high titres of anti-ds DNA correlated well with active nephritis (Davis et al., 1977), the high frequency of Raynaud's phenomenon associated with antiSm-and antiRNP-antibodies (Winn et al., 1979) and the incidence of disease symptoms in neonatal lupus (Provost , 1983).

Alarcon-Segovia et al. (1979 a) have shown that antiRNP antibodies can penetrate into MNC from patients with MCTD and SLE via their Fcγ receptors. They also suggested that the deficiency of T suppressor cells in patients with MCTD and SLE may be due to this penetration (Alarcon-Segovia et al., 1979 b). Therefore, a new immunological mechanism has been proposed to explain the disease pathology in SLE patients.

The use of autoantibodies has been suggested as a potent tool to investigate the function and structure of cellular constituent antigens. In addition, the study of their function may also enhance the understanding of autoimmune disease processes. My findings indicate that an inhibitory effect on protein synthesis

by specific autoantibodies (some ANAs) occurred only in cell-free systems. The inhibition was not related to any particular type of proteins or ANAs or to any one type of cell-free system. However, this effect could not be shown in intact cells because there was a limit to the efficiency of transfer of macromolecules into cells. From these results, it may be suggested that the amount of ANAs that would be needed for the suppression of protein synthesis in intact cells is relatively high and it is unlikely that an SLE patient would achieve such an amount of ANAs within MNC by Fcγ receptor uptake from the damage to the cell. Moreover, Alarcon-Segovia (1979 a) has reported the low incidence of intranuclear antibody-Fc receptor bearing MNC in SLE and suggested this to be due to the amount and heterogeniety of the ANAs these patients have. They also found that there was no correlation between the serum titre of antiRNP antibodies and the percent of MNC that had demonstrated intranuclear antibody in vivo.

Therefore, although a significant inhibitory effect of ANAs on protein synthesis has been established in cell-free systems, this is unlikely to contribute to the disease process in SLE patients.

REFERENCES.

ABELSON,J. (1979)
ANN.REV.BIOCHEM. 48, 1035

ABRAHAM,A.K.,OLSNES,S., AND PIHL,A. (1979)
FEBS.LETT., 101, 93

AGUTTER,P.S.,Mc.CALDIN,B., AND Mc.ARDLE,H.J. (1979)
BIOCHEM.J., 182, 811

ALARCON-SEGOVIA,D.,FISHBEING,E., AND ESTRADA-PARRA,S. (1975)
J.RHEUM., 2, 172

ALARCON-SEGOVIA,D., AND LLORENTE,L. (1983)
CLIN.EXP.IMMUNOL., 52, 365

ALARCON-SEGOVIA,D.,RUIZ-ARGUELLES,A., AND FISHBEIN,E. (1978)
NATURE, 271, 67

ALARCON-SEGOVIA,D.,RUIZ-ARGUELLES,A., AND FISHBEIN,E. (1979a)
CLIN.EXP.IMMUNOL., 35, 364

ALARCON-SEGOVIA,D.,RUIZ-ARGUELLES,A., AND LLORENTE,L. (1979b)
J.IMMUNOL., 122, 1855

ALGRANATI,I.D. (1980)
BIOCHEM.BIOPHYS.RES.COMMUN., 96, 54

ALLEN,R.W.,FERRONE,S., AND HOCH,J.A. (1982)
MOL.IMMUNOL., 19, 1127

ALONOSO,A.,FLYTZANIS,C.N.,SCHATZLO,U., et al. (1979)
EUR.J.BIOCHEM., 94, 601

ALOYO,V.J. (1979)
ANAL.BIOCHEM., 99, 161

ALSPAUGH,M.A. AND MADDISON,P.J. (1979)
ARTH.RHEUM., 22, 796

ANDERSSON,L.C.,GAHMBERG,C.G.,TEERENHOVI,L., AND VUOPIO,P. (1979)
INT.J.CANCER, 24, 717

ANTMAN,K.H. AND LIVINGSTON,D.M. (1980)
CELL, 19, 627

ASSELBERGS,F.M.,MEULENBERG,E. ,Van VENROOIJ,W.J., et al. (1980)
EUR.J.BIOCHEM. 109, 159

AUSTIN,S.A. AND CLEMENTS,M.J. (1980)
FEBS.LETT., 110, 1

AVIV,H. AND LEDER,P. (1972)
PROC.NATL.ACAD.SCI.USA., 69, 1408

BABICH,C.C.,HERISSE,J.,COURTOIS,G.,GALIBERT,F., et al. (1980)
NATURE, 287, 246

BACKENDORF,C.,OVERBEEK,G.P.,Van BOOM,J.H., et al. (1980)
EUR.J.BIOCHEM., 110, 599

BAKER,C.C.,HERISSE,J.,COURTOIS,G.,GALIBERT,F., et al. (1979)
CELL, 18, 569

BANGLIONI,C.,LENZ,J.R., AND MARONEY,P.A. (1978)
EUR.J.BIOCHEM., 92, 155

BANTLE,J.A.,MAXWELL,I.H., AND HAHN,W.E. (1976)
ANAL.BIOCHEM., 72, 413

BARADA,F.A.,ANDREWS,B.S.,DAVIES,J.S., AND TAYLOR,R.P. (1981)
ARTH.RHEUM., 24, 1236

BARALLE,F.E. (1983)
INTER.REV.CYTO., 81, 71

BARTH,R.K.,GROSS,K.W.,GREMKE,L.C., AND HASTIE,N.D. (1982)
PROC.NATL.ACAD.SCI.USA., 79, 500

BATHURST,I.C.,CRAIG,R.K., AND CAMPBELL,P.N. (1980)
BIOCHEM.J., 186, 561

BATHURST,I.C. AND SMITH,M.G. (1982)
BIOCHEM.BIOPHYS.ACTA, 699, 84

BAYER,A.L.,CHRISTENSEN,M.E.,WALKER,B.W., et al. (1977)
CELL, 11, 127

BEACHY,R.N. AND ZAITLIN,M. (1977)
VIROLOGY, 81, 160

BEIER,H.,MUNDRY,K.W., AND ISSINGER,O-G. (19809
INTERVIROLOGY, 14, 292

BENNE,R. AND HERSHEY,J.W.B. (1978)
J.BIOL.CHEM., 253, 3078

BENOIST,C. AND CHAMBON,P. (1981)
NATURE, 290, 304

BENVENISTE,K.,WILEZEK,J.,RUGGIERI,A., AND STERN,R. (1976)
BIOCHEMISTRY, 15, 830

BERGAN,N.A. (1978)
METH.CELL.BIOL., 20, 325

BHARGAVA,M.M. (1983)
BIOCHEM.BIOPHYS.ACTA, 740, 190

BLOCK,S.R..WINFIELD,J.B.,LOCKSHIR,M.D., et al. (1975)
AM.J.MED., 59, 533

BLUESTEIN,H.G. AND WOODS,V.L. (1982)
ARTH.RHEUM., 25, 773

BONALDO,M.F.,SANTELLI,R.V., AND LARA,F.J.S. (1979)
CELL, 17, 827

BONNER,W.M. AND LASKEY,R.A. (1974)
EUR.J.BIOCHEM., 46, 83

BOOGAARD,G. AND DIXON, G.H. (1983a)
EXPT.CELL.RES., 143, 175

BOOGAARD,G AND DIXON,G.H. (1983b)
EXPT.CELL.RES., 143, 191

BRAWERMAN,G. (1974)
ANN.REV.BIOCHEM., 43, 621

BRIMACOMBE,R.,STOFFLER,G., AND WITTMANN,H.G. (1978)
ANN.REV.BIOCHEM., 47, 217

BRUNELL,C. AND LELAY,M.N. (1979)
EUR.J.BIOCHEM., 99, 273

BUCHANAN,R.R.C.,VENABLES,P.J.W.,MORGAN,A., et al. (1983)
CLIN.EXPT.IMMUNOL., 51, 8

BURK,K.H.,DREWINKO,B.,TRUJILLO,J.M., AND AHEN,M.G. (1978)
CANCER.RES., 38, 2508

BUSCH,M.,REDDY,R.,ROTHBLUM,L., AND CHOI,Y.C. (1982)
ANN.REV.BIOCHEM., 51, 617

CAPECCHI,M.R.,von der HARR,R.A.,CAPECCHI,N.E., et al. (1977)
CELL, 12, 371

CALVET,J.P. AND PEDERSON,T. (1978)
J.MOL.BIOL., 122, 361

CARMICHAEL,G.C. AND Mc MASTER,G.K. (1980)
METH.ENZYMOL., 65, 380

CASKEY,C.T. (1980)
TRENDS.BIOCHEM.SCI., 5, 234

CASKEY,C.T.,BOSCH,L., AND KONECKI,D.S. (1977)
J.BIOL.CHEM., 252, 4435

CASPERSON,G.F. AND VOSS,E.W. (1983a)
MOL.IMMUNOL., 20, 573

CASPERSON,G.F. AND VOSS,E.W. (1983b)
MOL.IMMUNOL., 20, 581

CELMA,M.L. AND EHRENFELD,E. (1975)
J.MOL.BIOL., 98, 761

CHAN,L.,MEANS,A.R., AND O'MALLEY,B.W. (1973)
PROC.NATL.ACAD.SCI.USA., 70, 1870

CHIRGWIN,J.M.,BRZYBYLA,A.E.,Mac DONALE,R.J., et al. (1979)
BIOCHEMISTRY, 18, 5294

CHOI,Y.C. AND RO-CHOI,T.S. (1980)
CELL.BIOL., 3, 609

CHRISTIAN,C.L. (1982)
ARTH.RHEUM., 25, 887

CHU,L-Y. AND RHOADS,R.E. (1980)
BIOCHEMISTRY, 19, 184

CIVELLI,O.,VINCENT,A.,BURI,J.F., AND SCHERRER,K. (1976)
FEBS.LETT., 72, 71

CLARK,B. (1980)
TRENDS.BIOCHEM.SCI., 5, 207

CLEMENS,M.J.,SAFER,B.,MERRICK,W.C.,ANDERSON,W.F., et al. (1975)
PROC.NATL.ACAD.SCI.USA., 72, 1286

CLEMENS,M.J. AND VAQUERO,C.M. (1978)
BIOCHEM.BIOPHYS.RES.COMMUN., 83, 59

CLEMENS,M.J. AND WILLIAMS,B.R.G. (1978)
CELL, 13, 565

COCHRANE,C.G. AND KOFFLER,D. (1973)
ADV.IMMUNOL., 16, 185

COHEN,A.S.,REYNOLDS,W.E.,FRANKLIN,E.C.,KULKA,J.P., et al. (1971)
BULL.RHEU.DIS., 21, 643

CONTENT,J.,LEBLEU,B.,NUDEL,U.,ZILBERSTEIN,A., et al. (1975)
EUR.J.BIOCHEM., 54, 1

CONTENT,J.,LEBLEU,B., AND De CLERCQ,E. (1978)
BIOCHEMISTRY, 17, 88

CRICK,F. (1979)
SCIENCE, 204, 264

DASKAL,Y.,KOMAROMY,L., AND BUSCH,H. (1980)
EXPT.CELL.RES., 126, 39

DAVIS,P.,CUMMING,R.H., AND VERRIER-JONE,J. (1977)
CLIN.EXPT.IMMUNOL., 28, 226

DESHPANDE,A.K.,CHATTERJEE,B., AND ROY,A.K. (1979)
J.BIOL.CHEM., 254, 8937

DEVEN,B.,CHIN,D., AND BURNETT,E.V. (1978)
J.IMMUNOL.METH., 19, 187

DOEL,M.T. AND CAREY,N.H. (1976)
CELL, 8, 51

DOLBY,T.W.,DEVUONO,J., AND CROCE,C.M. (1980)
PROC.NATL.ACAD.SCI.USA., 77, 6027

DONIS-KELLER,H.,MAXAM,A.M., AND GILBERT,W. (1977)
NUCL.ACIDS.RES., 4, 2527

DOUVAS,A.S. (1982)
PROC.NATL.ACAD.SCI.USA., 79, 5401

DOUVAS,A.S.,STUMPH,W.E.,REYES,P., AND TAN,E.M. (1979)
J.BIOL.CHEM., 254, 3608

DOUVAS,A.S. AND TAN,E.M. (1981)
in "THE CELL NUCLEUS" BUSCH,H. ed., vol.VIII,p369,
ACADEMIC PRESS., NEW YORK.

DUBOIS,E.L. (1974)
in "LUPUS ERYTHEMATOSUS" 2nd.ed.,p484,UNIVERSITY OF SOUTHERN
CALIFORNIA PRESS.,LOS ANGELES,CALIFORNIA

DUGUID,J.R.,STEINER,D.F., AND CHICK,W.L. (1976)
PROC.NATL.ACAD.SCI.USA., 73, 3539

ECONOMIDIS,J.V. AND PEDERSON,T. (1982)
PROC.NATL.ACAD.SCI.USA., 79, 1467

EDMONDS,M.,NAKAZATO,H.,KORWEK,L., et al. (1976)
PROG.NUCLE.ACIDS.RES.MOL.BIOL., 19, 99

EDWARDS,P.A.W.,SMITH,C.M.,NEVILLE,M.A., AND O'HARE,M.J. (1982)
EUR.J.IMMUNOL., 12, 641

EILAT,D. AND TOTAN,C.H. (1982)
CLIN.EXPT.IMMUNOL., 49, 283

EILIOT,B.E. AND TAKACS,B.J. (1979)
J.IMMUNOL., 123, 543

EPSTEIN,P.,REDDY,R., AND BUSCH,H. (1981)
PROC.NATL.ACAD.SCI.USA., 78, 1562

EPSTEIN,P.,REDDY,R.,HENNING,D., AND BUSCH,H. (1980)
J.BIOL.CHEM., 255, 8901

ERDMANN,V.A. (1976)
PROG.NUCL.ACIDS.RES.MOL.BIOL., 18, 45

FAIFERMAN,I. AND POGO,A.O. (1975)
BIOCHEMISTRY, 14, 3808

FARRELL,P.J.,BALKOW,K.,HUNT,T.,JACKSON,R.J., et al. (1977)
CELL, 11, 187

FARRELL,P.J.,SEN,G.C.,DUBOIS,M.F.,RATNER,L., et al. (1978)
PROC.NATL.ACAD.SCI.USA., 75, 5893

FARRELL,P.B. AND TAN,E.M. (1983)
in "RECENT ADVANCES IN CLINICAL IMMUNOLOGY" p111, PITMANS,BATH.

FEINGLASS,E.J.,ARNELL,F.C.,DORSCH,L.A., et al. (1976)
MEDICINE, 55, 823

FELBER,B.R.,ORKIN,S.H., AND HAMER,D.H. (1982)
CELL, 29, 895

FILIPOWICZ,W. AND HAENNI,A-L. (1979)
PROC.NATL.ACAD.SCI.USA., 76, 3111

FORCHHAMMER,J.,JACKSON,E.N., AND YANOTSKY,C. (1972)
J.MOL.BIOL., 71, 687

FRANCOCUR,A.M. AND MATHEWS, M.B. (1982)
PROC.NATL.ACAD.SCI.USA., 79, 6772

FRANCOISTRON,J.L.,BARREIRA,M.C.R-A., AND INSERM,P.L. (1982)
CLIN.EXPT.IMMUNOL., 49, 481

FRESNO,M.,CARRASCO,L., AND VÁZQUEZ,D. (1976)
EUR.J.BIOCHEM., 68, 335

FUCHS,J.P. AND JACOB,M. (1979)
BIOCHEMISTRY, 18, 4202

FURUSAWA,M. (1980)
INT.REV.CYTO., 62, 29

GABRIELLI,J.B. AND BAGLIONI,C. (1977)
NATURE, 296, 529

GAHMBERG,C.G. AND ANDERSSON,L.C. (1981) SEMIN. HEMATOL., 18, 72

GALLINARO-MATRINGE,H. AND JACOB,M. (1973)
FEBS.LETT., 36, 105

GALLINARO-MATRINGE,H.,STÉVENIN,J., AND JACOB,M. (1975)
BIOCHEMISTRY, 14, 2547

GAMBINO,R.,METAFORA,S.,FELICETTE,L., AND RAISMAN,J. (1973)
BIOCHEM.BIOPHYS.ACTA., 312, 377

GANOZA,M.C.,SULLIVAN,P.,CUNNINGHAM,C.,HADER,P., et al. (1982)
J.BIOL.CHEM., 257, 8228

GARRELS,J.I. AND GIBSON,W. (1979)
CELL, 9, 793

GATTIONI,R.,STÉVENIN,J.,DEVILLIERS,G., AND JACOB,M. (1978)
FEBS.LETT., 90, 318

GEORGIEV,G.P. (1961)
BIOKHIMIYA, 26, 1095

GEORGIEV,G.P. (1974)
in "THE CELL NUCLEUS" BUSCH,H. ed., vol.III,p67,
ACADEMIC PRESS.,NEW YORK.

GEORGIEV,G.P.,RYSKOV,A.P.,COUTELLE,Ch., et al. (1972)
BIOCHEM.BIOPHYS.ACTA., 259, 259

GIBBONS,J.J.,AUGUSTYNEK,D.,TSAI,C.C., AND ROADMAN,S.T. (1982)
MOL.IMMUNOL., 19, 765

GILOH,H.,SCHOCHOT,L., AND MAGER,J. (1975)
BIOCHEM.BIOPHYS.ACTA., 414, 309

GODFREY,W.,DOE,B., AND WOFSY,L. (1983)
PROC.NATL.ACAD.SCI.USA., 80, 2267

GOELET,P. AND KARN,J. (1982)
J.MOL.BIOL., 154, 541

GOELET,P.,LOMONOSSOFF,G.P.,BUTLER,P.J.G., et al. (1982)
PROC.NATL.ACAD.SCI.USA., 79, 5818

GOLDENBERG,S. AND SCHERRER,K. (1981)
FEBS.LETT., 133, 213

GOLDMAN,M. (1968)
in "FLUORESCENCE ANTIBODY METHODS"ACADEMIC PRESS.,NEW YORK.

GRILL,L.K.,SUN,J.D., AND KANDEL,J. (1976)
BIOCHEM.BIOPHYS.RES.COMMUN., 73, 149

GROSSCHEDL,R.,WASYLYK,B.,CHAMBON,P., AND BIRNSTIEL,M.L. (1981)
NATURE, 294, 178

GRUNBERG-MANAGO,M.,BUCKINGHAM,R.H.,COOPERMAN,B.S.,et al. (1978)
SYMP.SOC.GEN.MICROBIOL., 28, 25

GURDON,J.B.,WOODLAND,H.R., AND LINGREL,J.B. (1974)
DEVL.BIOL., 39, 125

HALBERT,S.P.,KARSH,J., AND ANKEN,M. (1981)
J.LAB.CLIN.MED., 97, 97

HALEGOUA,S.,HIRASHIMA,A.,SEKIZAWA,J., AND INOUYA,E. (1976)
EUR.J.BIOCHEM., 69, 163

HARDIN,J.A.,RAHN,D.R.,SHEN,C.,LERNER,M., et al. (1982)
J.CLIN.INVEST., 70, 141

HELLUNG-LARSEN,P. (1977)
in "LOW M.W. RNA COMPONENT IN EUKARYOTIC CELLS" FADL'S
FORLAG,COPENHEGEN.

HENDRICK,J.P.,WOLIN,S.L.,RINKE,J.,LERNER,M., et al. (1981)
MOL.CELL.BIOL., 1,1138

HERMAN,R.,ZIEVE,G.,WILLIAMS,J.,LENK,R., et al. (1976)
PROG.NUCL.ACIDS.RES.MOL.BIOL., 19, 379

HERNANDEZ,N. AND KELLER,W. (1983)
CELL, 35, 89

HESS,E.V. (1982)
ARTH.RHEUM., 25, 857

HEYWOOD,S.M. AND KENNEDY,D.S. (1976)
BIOCHEMISTRY, 15, 3314

HINTERBERGER,M.,PETTERSSON,I., AND STEITZ,J.A. (1983)
J.BIOL.CHEM., 258, 2604

HIRANYAVASIT,W. AND KUSAMRAN,T. (1983)
FEBS.LETT., 152, 35

HIRE,H.W. (1967)
METH.ENZYMOL., XI, 199

HOFER,E. AND DARNELL,J.E. (1981)
CELL, 23, 585

De HORATIUS,R.J. (1982)
ARTH.RHEUM., 25, 828

De HORATIUS,R.J.,PILLARISETTY,R.,MESSNER,R.P., et al. (1975)
J.CLIN.INVEST., 56, 1149

HOVANESSIAN,A.G. AND KERR,I.M. (1978)
EUR.J.BIOCHEM., 84, 149

HUDSON,L AND HAY,F.C. (1980)
in "PRACTICAL IMMUNOLOGY" 2nd ed.,BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD,ENGLAND.

HUEZ,G.,MARBAIX,G.,WEINBERG,E.,GALLWITZ,D., et al. (1977)
BIOCHEM.SOC.TRANS., 5, 936

HUNT,T. (1980)
TRENDS.BIOCHEM.SCI., 5, 178

HUNTER,A.R.,FARRELL,P.J.,JACKSON,R.J., AND HUNT,H. (1977)
EUR.J.BIOCHEM., 75, 149

HUNTER,T.R.,HUNT,T.,KNOWLAND,J., AND ZIMMEN,D. (1976)
NATURE, 260, 759

HUNTER,T.R.,JACKSON,R., AND ZIMMERN,D. (1983)
NUCL.ACIDS.RES., 11, 801

IGARASHI,K.,HASHIMOTO,S.,MIYAKE,A.,KASHIWAGI,K., et al. (1982)
EUR.J.BIOCHEM., 128, 597

IGARASHI,K.,KASHIWAGI,K.,AOKI,R.,KOJIMA,M., et al. (1979)
BIOCHEM.BIOPHYS.RES.COMMUN., 91, 440

ISONO,S. AND ISONO,K. (1975)
EUR.J.BIOCHEM., 56, 15

JACOB,J. AND DANIELLI,G.A. (1972)
CELL DIFFER., 1, 119

JACOB,J.,DEVILLIERS,G.,FUCH,J-P.,GALLINARO,H., et al. (1981)
in "THE CELL NUCLEUS" BUSCH,H.,ed.,vol VIII,p193,ACADEMIC PRESS.,NEW YORK.

JACOBSON,M.F. AND BALTIMORE,D. (1968)
PROC.NATL.ACAD.SCI.USA., 61, 77

JACOBSON,A.AND FAVREAU,M. (1983)
NUCL.ACIDS.RES., 11, 6353

JAGUS,R.,ANDERSON,W.F., AND SAFER,B. (1981)
PROG.NUCL.ACID.RES.MOL.BIOL., 25, 127

JAY,G.,ABRAMS,W.R., AND KAEMPFER,R. (1974)
BIOCHEM.BIOPHYS.RES.COMMUN., 60, 1357

JOHNSTON,A. AND THORPE,R. (1982)
in "IMMUNOCHEMISTRY IN PRACTICE" p89, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, LONDON.

KAHN,A.,COTTREAU,D.,DAEGELEN,D., AND DREYFUR,J-C. (1981)
EUR.J.BIOCHEM., 116, 7

KANCHISA,T.,KITAZUME,Y.,IKUTA,K., AND TANAKA,Y. (1977)
BIOCHEM.BIOPHYS.ACTA., 475, 501

KEARNEY,J.,RADBRUCH,A.,LIESEGANG,B., AND RAJEWSKY,K. (1979)
J.IMMUNOL., 123, 1548

KERR,I.M. AND BROWN,R.E. (1978)
PROC.NATL.ACAD.SCI.USA., 75, 256

KERR,I.M.,BROWN,R.E., AND BALL,L.A. (1974)
NATURE, 250, 57

KERR,I.M.,BROWN,R.E.,CLEMENTS,M.J., AND GILBERT,C.S. (1976)
EUR.J.BIOCHEM., 69, 551

KERR,I.M.,BROWN,R.E., AND HOVANESSIAN,A.G. (1977)
NATURE, 268, 540

KINLAW,C.S.ROBBERSON,B.L., AND BERGET,S.M. (1983)
J.BIOL.CHEM., 258, 7181

KINNIBURGH,A.J. AND ROSS,J. (1979)
CELL, 17, 915

KINNIBURGH,A.J. AND MARTIN,T.E. (1976)
BIOCHEM.BIOPHYS.RES.COMMUN., 73, 718

KISH,V.M. AND PEDERSON,T. (1977)
PROC.NATL.ACAD.SCI.USA., 74, 1426

KLEIN,E.,BEN-BASAT,H.,NEUMANN,H.,RALPH,P., et al. (1976)
INT.J.CANCER, 18, 421

KNOWLAND,J. (1974)
GENETICS, 78, 383

KOFFLER,D.,BIESECKER,G., AND KATZ,S.M. (1982)
ARTH.RHEUM., 25, 858

KOFFLER,D.,CAN,R.I.,ANGELLO,V.,THOBURN,R., et al. (1971)
J.EXPT.MED., 134, 294

KOIKE,T.,NAGRAWA,R.,NAGATA,N., AND SHIRAI,T. (1982)
IMMUNOL.LETT., 4, 93

KOLE,R. AND ALTMAN,S. (1979)
PROC.NATL.ACAD.SCI.USA., 76,3795

KOZAK,M. (1978)
CELL, 15, 1109

KOZAK,M. (1980a)
CELL, 19, 79

KOZAK,M. (1980b)
CELL, 22, 1

KOZAK,M. AND SHATKIN,A.Y. (1978)
CELL, 13, 201

KRAUSE,M.O. AND RINGUETTE,M.J. (1977)
BIOCHEM.BIOPHYS.RES.COMMUN., 76, 796

KRAUSE,M.O. AND RINGUETTE,M.J. (1982)
in "GENETIC EXPRESSION IN THE CELL CYCLE" PADILLA,G.M. AND McCARTY,Sr.K.S., ed.,p151,ACADEMIC PRESS.,NEW YORK.

KRYSTOSEK,A.,CAWTHON,M.L., AND KABAT,D. (1975)
J.BIOL.CHEM., 250, 6077

KÜHN,B.,VILLRINGER,A.,FALK,H., AND HEINRICH,P.C. (1982)
EUR.J.BIOCHEM., 126, 181

KÜHN,B., et al. (1983)
EMBO.J., 2, 727

KURKINEN,M. (1981)
FEBS.LETT., 124, 79

KURATA,N. AND TAN,E.M. (1976)
ARTH.RHEUM., 19, 574

LAEMMLI,U.K. (1970)
NATURE, 227, 680

LAHITA,R.G.,BRADLOW,L.,FISHMAN,J., AND KUNKEL,H.G. (1982)
ARTH.RHEUM., 25, 843

LANGFORD,C.J. AND GALLWITZ,D. (1983)
CELL, 33, 519

LASKEY,R.A. AND MILLS,A.D. (1975)
EUR.J.BIOCHEM., 56, 335

LEE-HUANG,S.,SIERRA,J.M.,NARANJO,R., et al. (1977)
ARCH.BIOCHEM.BIOPHYS., 180, 276

LENK,R.P.,MAIZEL,Jr.J.V., AND CROUCH,R.J. (1982)
EUR.J.BIOCHEM., 121, 475

LERNER,M.R.,ANDREWS,N.C.,MILLER,G., AND STEITZ,J.A. (1981a)
PROC.NATL.ACAD.SCI.USA., 78, 805

LERNER,M.R.,BOYLE,J.A.,HARDIN,J.A., AND STEITZ,J.A. (1981b)
SCIENCE, 211, 400

LERNER,M.R.,BOYLE,J.A.,MOUNT,S.M.,WOLIN,S.L., et al. (1980)
NATURE, 283, 220

LERNER,E.A.,LERNER,M.R.,HARDIN,J.A., et al. (1982)
ARTH.RHEUM., 25, 761

LERNER,M.R. AND STEITZ,J.A. (1979)
PROC.NATL.ACAD.SCI.USA., 76, 5495

LERNER,M.R. AND STEITZ,J.A. (1981)
CELL, 25, 298

LeROY,E.C. (1982)
ARTH.RHEUM., 25, 889

Le STOURGEON,W.M.,BEJER,A.L.,CHRISTENSEN,M.E., et al. (1978)
COLD.SPRING.HARBOR.SYMP.QUANT.BIOL., 42, 885

LEVIN,D.,RANU,R.S.,ERNST,V.,TRACHSEL,H., et al. (1977)
PROC.FEBS.MEET.11th., 42, 27

LEWIN,B. (1980)
in "GENE EXPRESSION" vol.2, WILLEY, NEW YORK.

LEWIS,J.,FALCOFF,E., AND FALCOFF,R. (1978)
EUR.J.BIOCHEM., 86, 497

LIN,W. AND KARAMATSU,H. (1983)
ANAL.BIOCHEM., 128, 302

LODISH,H.F. (1976)
ANN.REV.BIOCHEM., 45, 39

LODISH,H.F.,HOUSMAN,D., AND JACOBSEN,M. (1971)
BIOCHEMISTRY, 10, 2348

LODISH,H.F. AND NATHAN,D.G. (1972)
J.BIOL.CHEM., 247, 7822

LOMEDICO,P.T. AND SAUNDERS,G.F. (1976)
NUCL.ACIDS.RES., 3, 381

LOWRY,O.H.,ROSEBROUGH,N.J.,FARR,A.L., AND RANDALL,R.J. (1951)
J.BIOL.CHEM., 193, 265

LOZZIO,C.B. AND LOZZIO,B.B. (1975)
BLOOD, 45, 321

LUKANIDIN,E.M.,ZALMANZON,E.S.,KOMAROMI,L., et al. (1972)
NATURE, 238, 193

Mac GALLIVRAY,A.J.,CARROLL,A.R.,DAHI,S., et al. (1982)
FEBS.LETT., 141, 139

MADDISON,P.J. AND REICHLIN,M. (1979)
ARTH.RHEUM., 22, 858

MADDISON,P.J.,PROVOST,T.T., AND REICHLIN,M. (1981)
MEDICINE, 60, 87

MAGEE,W.E. AND MILLER,O.V. (1972)
NATURE, 235, 339

MALCOLM,D.B. AND SOMMERVILLE,J. (1977)
J.CELL.SCI., 24, 143

MANIATIS,T.,FRITSCH,E.F., AND SAMBROOK,J. (1982)
in "MOLECULAR CLONING" A LABORATORY MANUAL, CSH.

MANNIK,M. (1982)
ARTH.RHEUM., 25, 783

MARCH,S.C.,PARIKH,I., AND CUATRECASAS,P. (1974)
ANAL.BIOCHEM., 60, 149

MARCHALONIS,J.J.,ATWELL,J.L., AND HAUSTEIN,D. (1974)
BIOCHEM.BIOPHYS.ACTA., 351, 99

MARCU,K. AND DUDOCK,B. (1974)
NUCL.ACIDS.RES., 1, 1385

MARTIN,T.,BILLING,P.,PULLMAN,J.,STEVENS,B., et al. (1978)
COLD.SPRING.HARBOR.SYMP.QUANT.BIOL., 42, 899

MASON,D.Y. AND SAMMONS,R. (1978)
J.CLIN.PATHOL., 31, 454

MATHEWS,M.B. (1971)
FEBS.LETT., 15, 201

MATHEWS,M.B. AND OSBORN,M. (1974)
BIOCHEM.BIOPHYS.ACTA., 340, 147

MATSUOKA,Y.,MOORE,G.E.,YAGI,Y., AND PRESSMAN,D. (1967)
P.S.E.B.M., 125, 1246

Mc.CLUSKEY,R.T. (1982)
ARTH.RHEUM., 25, 867

MECHLER,B. AND RABBITTS,T.H. (1981)
J.CELL.BIOL., 88, 29

MILLER,T.E.,HUANA,C-Y., AND POGO,A.O. (1978)
J.CELL.BIOL., 76, 675

MILLER,M.J. AND WAHBA,A.J. (1974)
J.BIOL.CHEM., 249, 3808

MILMAN,G.,GOLDSTEIN,J.,SCOLNIK,E., AND CASKEY,T. (1969)
PROC.NATL.ACAD.SCI.USA., 63, 183

MISHELL,B.B. AND SHIIGI,S.M. (1980)
in "SELECTED METHODS IN CELLULAR IMMUNOLOGY"
FREEMAN W.H.COMPANY.

MOFFETT,R.B. AND WEBB,T.E. (1981)
BIOCHEMISTRY, 20, 3253

MOFFETT,R.B. AND WEBB,T.E. (1983)
BIOCHEM.BIOPHYS.ACTA., 740, 231

MOLGAARD,H.V.,WEIR,L.,KENTEN,J.,CRAMER,F., et al. (1981)
BIOCHEMISTRY, 20, 4467

MONTELL,C.,FISHER,E.F.,CARUTHERS,M.H., AND BERK,A.J. (1982)
NATURE, 295, 380

MONTELL,C.,FISHER,E.F.,CARUTHERS,M.H., AND BERK,A.J. (1983)
NATURE, 305, 600

MOUNT,S.M. (1982)
NUCL.ACIDS.RES., 10, 459

MOUNT,S.M.,PETTERSSON,I.,HINTERBERG,M., et al. (1983)
CELL, 33, 509

MUKHERJEE,A.K. AND SARKAR,S. (1981)
MOL.BIOL.REP., 8, 51

MUNOZ,R.F. AND DARNELL,J.E. (1974)
CELL, 2, 247

NAORA,H. (1979)
INTER.REV.CYTO., 56, 255

NAORA,H. AND DEACON,W.J. (1981)
DIFFERENTIATION, 18, 125

NAORA,H.,DEACON,N.J., AND FRY,K.E. (1979)
J.THEOR.BIOL., 80, 205

NEVINS,J.R. (1979)
J.MOL.BIOL., 130, 493

NEVINS,J.R. (1983)
ANN.REV.BIOCHEM., 52, 441

NEVINS,J.R.,BLANCHARD,J.M., AND DARNELL,J.E. (1980)
J.MOL.BIOL., 144, 377

NEVINS,J.R. AND DARNELL,J.E. (1978)
CELL, 15, 1477

NORTHEMANN,W.,GROSS,W.,SCHEURLEN,M., AND HEINRICH,P.C. (1978)
BIOCHEM.BIOPHYS.ACTA., 519, 406

NOTMAN,D.D.,KURATA,N., AND TAN,E.M. (1975)
ANN.INT.MED., 83, 464

NOYES,B.E.,MEVARECH,M.,STEIN,R., AND AGARWAL,K.L. (1979)
PROC.NATL.ACAD.SCI.USA., 76, 1770

NUDEL,U.,SOREZ,H.,LITTAUER,U.Z.,MARBAIX,G., et al. (1976)
EUR.J.BIOCHEM., 64, 115

OCHOA,S. AND HARO,C. (1979)
ANN.REV.BIOCHEM., 48, 549

O'FARRELL,P.H. (1975)
J.BIOL.CHEM., 250, 4007

ORCHINNIKEV,Yu.A.,ALAKHOV,Yu.B.,BUNDULIS,Yu.p., et al. (1982)
FEBS.LETT., 139, 130

ORKIN,S.H.,KAZAZIAN,H.H.,ANTONARKIS., S.E., et al. (1982)
NATURE, 296, 627

OSTERBERG,H.H.,ALLEN,J.K., AND FINCH,C.E. (1975)
BIOCHEM.J., 147, 367

OUCHTERLONY,D. (1958)
PROG.ALLERGY, 5, 1

PADGETT,R.A.,HARDY,S.F., AND SHARP,P.A. (1983a)
PROC.NATL.ACAD.SCI.USA., 80, 5230

PADGETT,R.A.,MOUNT,S.M.,STEITZ,J.A., AND SHARP,P.A. (1983b)
CELL, 35, 101

PAGANO,R.E. AND TAKEICHI,M. (1977)
J.CELL.BIOL., 73, 531

PAGANO,R.E. AND WEINSTEIN,J.N. (1978)
ANN.REV.BIOPHYS.BIOENG., 7, 435

PALATNIK,E.M.,STORTI,R.V.,CAPONE,A.R., AND JACOBSON,A. (1980)
J.MOL.BIOL., 141, 99

PALMITER,R.D. (1974)
BIOCHEMISTRY, 13, 3606

PANTOJA,S.,ZARRAGA,A.M.,VILLANNEVA,J., AND KRAUSKOPF,M. (1981)
IRCS.MED.SCI,, 9, 651

PAOLETTI,E.,LIPIRSKAS,B.R., AND PANICALI,D. (1980)
J.VIROL., 33, 208

PAPAHADJOPOULOS,D.,NIR,S., AND OHKIS,S. (1973)
BIOCHEM.BIOPHYS.ACTA., 266, 561

PAPALIAN,M.,LAFER,E.,WONG,R., AND STOLLAR,B.D. (1980)
J.CILN.INVEST., 65, 469

PEDERSON,T. (1980)
AMER.SCI., 69, 76

PELHAM,H.R.B. (1978)
NATURE, 272, 469

PELHAM,H.R.B. AND JACKSON,R.T. (1976)
EUR.J.BIOCHEM., 67, 247

PERRY,R.P. (1976)
ANN.REV.BIOCHEM., 45, 605

PHILLIPS,P.E. (1975)
ANN.INTERN.MED., 83, 709

PINCUS,T. (1982)
ARTH.RHEUM., 25, 847

PISETSKY,D.S. AND PETERS,D.V. (1981)
J.IMMUNOL.METH., 41, 187

PODUSLO,J.F. AND RODBARD,D. (1980)
ANAL.BIOCHEM., 101, 394

POSTE,G. (1980)
in "LIPOSOME IN BIOLOGICAL SYSTEMS", GREGORIADIS,G. AND
ALLISON,A.C.,eds., p101, JOHN WILEY & SONS, LTD.

POSTE,G. AND PAPAHADJOPOULOS,D. (1978)
ANN.N.Y.ACAD.SCI., 308, 164

PRIBNOW,D. (1979)
in "BIOLOGICAL REGULATION AND DEVELOPMENT"
GOLDENBERG,R.,ed.,vol.1,p219,PLENUM,NEW YORK.

PROUD,C.G. AND PAIN,V.M. (1982)
BIOCHEM.SOC.TRANS., 10, 89

PROVOST,T.T. (1979)
J.INVEST.DERMATOL., 72,110

PROVOST,T.T. (1983)
ARCH.DERMATOL., 119, 619

PROVOST,T.T. AND REICHLIN,M. (1981)
J.AM.ACAD.DERMATOL., 4, 84

QUINLAN,T.,KINNIBURGH,A., AND MARTIN,T. (1977)
J.BIOL.CHEM., 252, 1156

RANU,R.S. AND BHALA,K.K. (1981)
BIOCHEM.BIOPHYS.RES.COMMUN., 102, 30

RAO,M.S.,BLACKSTONE,M., AND BUSCH,H. (1977a)
BIOCHEMISTRY, 16, 2756

RAO,M.S.,HIRSH,F.,WU,B.C.,SPOHN,W.H.. et al. (1977b)
MOL.CELL.BIOCHEM., 15, 3

RATNER,L.,WIEGAND,R.C.,FARRELL,P.J.,SEN,G.C., et al. (1978)
BIOCHEM.BIOPHYS.RES.COMMUN., 81, 947

RAVEL,M. AND GRONER,Y. (1978)
ANN.REV.BIOCHEM., 47, 1079

RECHSTEINER,M.C. (1975)
EXPT.CELL.RES., 93, 487

REDDY,R. AND BUSCH,H. (1981)
in "THE CELL NUCLEUS" BUSCH,H. ed., vol.8, p261,
ACADEMIC PRESS., NEW YORK.

REDDY,R.,HENNING,D., AND BUSCH,H. (1979)
J.BIOL.CHEM., 254, 11097

REDDY,R..HENNING,D., AND BUSCH,H. (1980)
J.BIOL.CHEM., 255, 7029

REDDY,R.,HENNING,D., AND BUSCH,H. (1981)
J.BIOL.CHEM., 256, 3532

REDDY,R.,TAN,E.M.,HENNING,D.,NOHGA,K., et al. (1983)
J.BIOL.CHEM., 258, 1383

REICHLIN,M. (1981)
CLIN.EXPT.IMMUNOL., 44, 1

REYES,P.A. AND TAN,E.M. (1977)
J.EXP.MED., 145, 749

RINGUETTE,M.,LIE,W.C.,JAY,E.,YU,K.K-Y., et al. (1980)
GENE, 8, 211

RINKE,J. AND STEITZ,J.A. (1982)
CELL, 29, 149

ROBERTS,B.E.,GORECKI,M.,MULLIGAN,R.C., et al. (1975)
PROC.NATL.ACAD.SCI.USA., 72, 1922

ROBERTS,B.E. AND PATTERSON,B.M. (1973)
PROC.NATL.ACAD.SCI.USA., 70, 2330

ROBERTS,B.E.,PATTERSON,B.M., AND SPERLING,R. (1974)
VIROLOGY, 59, 307

ROBERTSON,H.D. AND MATHEWS,M.B. (1973)
PROC.NATL.ACAD.SCI.USA., 70, 225

RO-CHOI,T.S. AND BUSCH,H. (1974)
in "THE CELL NUCLEUS" BUSCH,H. ed., vol.8, p151,
ACDEMIC PRESS., NEW YORK.

ROGER,S.J. AND WALL,R. (1980)
PROC.NATL.ACAD.SCI.USA., 77, 1877

ROSA,M.D.,GETTLIEB,E.,LERNER,M.R., AND STEITZ,J.A. (1981)
MOL.CELL.BIOL., 1, 785

ROSEN,H.,KNOLLER,S., AND KAEMPFER,R. (1981)
BIOCHEMISTRY, 20, 3011

ROSEN,J.M.,WOO,S.L.C.,HOLDER,J.M.,MEANS,A.R., et al. (1975)
BIOCHEMISTRY, 14, 69

RUBIN,J.R.,MORIKAWA,K.,NYBORG,J.,LaCOUR,T.F.M., et al. (1981)
FEBS.LETT., 129, 177

RUDERMAN,J.V. AND GROSS,P.R. (1975)
DEVL.BIOL., 36, 286

RUIZ,N. AND KRAUSKOPF,M. (1980)
LIFE.SCI., 27, 2359

RUTHURFORD,T.,CLEGG,J.B.,HIGGS,D.R.,JONES,R.W., et al. (1981)
PROC.NATL.ACAD.SCI.USA., 78, 348

RYMAN,B.E. AND TYRRELL,D.A. (1980)
ESSAYS.BIOCHEM., 16, 49

SABAHARVAL,U.K.,FONG,S.,HOCH,S.,COOK,R., et al. (1983)
CLIN.EXPT.IMMUNOL., 51, 317

SAFER,B. AND ANDERSON,F.W. (1978)
CRIT.REV.BIOCHEM., 5, 361

SAMARINA,O.P. (1964)
BIOCHEM.BIOPHYS.ACTA., 91, 688

SAMARINA,O.P. AND KRICHEVSKAYA,A.A. (1981)
in "THE CELL NUCLEUS" BUSCH,H. ed., vol.IX, p1,
ACADEMIC PRESS., NEW YORK.

SAMARINA,O.P.,LUKANIDIN,E.M.,MOLNAR,J. AND GEORGIEV,G.P. (1968)
J.MOL.BIOL., 33, 251

SARMA,M.H.,BEACH,T.A., AND CHATTERJEE,N.K. (1978)
BIOCHEM.BIOPHYS.RES.COMMUN., 82, 384

SCHAFFNER,W. AND WEISSMANN,C. (1973)
ANAL.BIOCHEM., 56, 502

SCHEELE,G. AND BLACKBURN,P. (1979)
PROC.NATL.ACAD.SCI.USA., 76, 4898

SCHLEGEL,R.A. AND RECHSTEINER,M.C. (1978)
METH.CELL.BIOL., 20, 341

SCHMECKPEPER,B.J.,CORY,S., AND ADAMS,J.M. (1974)
MOL.BIOL.REPORTS, 1, 355

SCHRAGER,M.A. AND ROTHFIELD,N.F. (1976)
J.CLIN.INVEST., 57, 221

SCHRIER,W.H.,REDDY,R., AND BUSCH,M. (1982)
CELL.BIOL.INT.REP., 6, 925

SCHUR,P.H. (1975)
CLIN.RHEUM.DIS., 1, 519

SCOLNICK,E.M. AND CASKEY,C.T. (1969)
PROC.NATL.ACAD.SCI.USA., 64, 1235

SCOPELITIS,E.,BUIADO,J.J., AND ALSPAUGH,M.A. (1980)
ARTH.RHEUM., 23, 287

SEKERIS,C.E. AND GUIALIS,A. (1981)
in "THE CELL NUCLEUS" BUSCH,H. ed., vol.VII, p247,
ACADEMIC PRESS., NEW YORK.

SHAFRITZ,D.A.,WEINSTEIN,J.A.,SAFER,B., et al. (1976)
NATURE, 261, 291

SHARP,G.C. (1982)
ARTH.RHEUM., 25, 757

SHARP,P.A.,SUGDEN,B., AND SAMBROCK,J. (1973)
BIOCHEMISTRY, 12, 3055

SHATKIN,A.J. (1976)
CELL, 9, 645

SHIBATA,H.,RO-CHOI,T.S.,REDDY,R.,CHOI,Y.C., et al. (1975)
J.BIOL.CHEM., 250, 3909

SHINE,J. AND DALGARNO,L. (1975)
NATURE, 254, 34

SHUTTLEWORTH,J.,MORSER,J., AND BURKE,D. (1982)
BIOCHEM.BIOPHYS.ACTA., 698, 1

SKINNER,M.K. AND GRISWOLD,M.D. (1983)
BIOCHEM.J., 209, 281

SLEGERS,H.,De HERDT,E., AND KONDO,M. (1981)
EUR.J.BIOCHEM., 117, 111

SMITH,A.E. AND CARRASCO,L. (1978)
INT.REV.BIO., 18, 261

SMITH,J.H.,SUBBARAO,M.N., AND ELICEIRI,G.L. (1983)
J.CELLULAR.PHYSIO., 114, 1

SONENBERG,M.,RUPPRECHT,K.M.,HECHT,S.M., AND SHATKIN,A.J. (1979)
PROC.NATL.ACAD.SCI.USA., 76, 4345

SPIRIN,A.S. (1969)
EUR.J.BIOCHEM., 10, 20

SPRITZ,R.A.,JAGADESWARAN,P.,CHOWDARY,P.V.,et al. (1981)
PROC.NATL.ACAD.SCI.USA., 78,2455

STEITZ,J.A. AND JAKES,K. (1975)
PROC.NATL.ACAD.SCI.USA., 72, 4734

STEITZ,J.A.,RINKE,W.J.,PETTERSSON,I.,MOUNT,S.M., et al. (1982)
COLD.SPRING.HARB.SYMP.QUANT.BIOL., 47, 893

STÉVENIN,J.,GALLINARO-MATRINGE,H.,GATTIONI,R., et al. (1977)
EUR.J.BIOCHEM., 74, 589

STÉVENIN,J.,GATTONI,R.,DEVILLIERS,G., AND JACOB,M. (1979)
EUR.J.BIOCHEM., 95, 593

STEWART,P.R. AND LETHAM,D.S. (1977)
in "THE RIBONUCLEIC ACID" 2nd. ed., SPRINGER-VERLAG, NEW YORK,
HEIDELBERG.

STOLTZFUS,C.M. AND DANE,R.W. (1982)
J.VIROL., 42, 918

SUZUKI,H. (1977)
J.BIOCHEM., 85, 251

SUZUKI,H. (1981)
J.BIOCHEM., 90, 1047

SUZUKI,H.,BESSHO,E., AND NAKAMURA,S. (1980)
FEBS.LETT., 110, 333

SVEC,K.H. AND ALLEN,S.T. (1970)
SCIENCE, 170, 550

SWAAK,A.J.,GROENWALD,J.,AURDEN,L.A., et al. (1982)
ANN.RHEUM.DIS., 41, 388

SZEKELY,M. (1980)
in "FROM DNA TO PROTEIN, THE TRANSFER OF GENETIC INFORMATION"
THE MACMILLAN PRESS LTD.,LONDON AND BASINGSTOKE.

SZER,W.,HERMOSO,J.M., AND LEFFLER,S. (1975)
PROC.NATL.ACAD.SCI.USA., 72, 2325

SZOKA,F.,JACOBSON,K., AND PAPAHADJOPOULOS,D. (1979)
BIOCHEM.BIOPHYS.ACTA., 551, 295

SZOKA,F.,MAGNUSSON,K.E.,WOJCIESZYN,J.,HOU,Y., et al. (1981)
PROC.NATL.ACAD.SCI.USA., 78, 1685

SZOKA,F. AND PAPAHADJOPOULOS,D. (1978)
PROC.NATL.ACAD.SCI.USA., 75, 4194

SZYBALSKI,W. AND SZYBALSKI,E. (1971)
PROCEDURES.NUCL.ACID.RES., 2, 311

TAKANO,M.,AGRIS,P.E., AND SHARP,G.C. (1980)
J.CLIN.INVEST., 65, 1449

TAKANO,M.,GOLDEN,S.S.,SHARP,G.C., AND AGRIS,P.E. (1981)
BIOCHEMISTRY, 20, 5929

TALAL,N.,DAUPHINIE,M.J. AND WOLFREY,D. (1982)
ARTH.RHEUM., 25, 834

TAN,E.M. (1982)
ARTH.RHEUM., 25, 753

TAN,E.M.,FRITZLER,M.J.,Mc DOUGAL,J.S., et al. (1982)
ARTH.RHEUM., 25, 1003

TAN,E.M.,ROBINSON,J., AND ROBITAILE,P. (1976)
SCAND.J.IMMUNOL., 5, 811

TATE,W.P.,CASKEY,C.T. AND STOFFLER,G. (1975)
J.MOL.BIOL., 93, 375

TAYLOR,J.M. (1979)
ANN.REV.BIOCHEM., 48, 681

TAYLOR,J.M. AND SCHIMKE,R.T. (1973)
J.BIOL.CHEM., 248, 7661

TEPPO,A. (1981)
CLIN.CHEM., 27, 1341

TEPPO,A.M.,GRIPENBERG,M.,KURTI,P.,BAKLEIN,K., et al. (1982)
SCAN.J.IMMUNOL., 15, 1

TOWBIN,H.,STACHELIN,T., AND GORDON,J. (1979)
PROC.NATL.ACAD.SCI.USA., 76, 4350

TRACHSEL,H.,ERNI,B.,SCHREIER,M., AND STAEHELIN,M. (1977)
J.MOL.BIOL., 116, 755

TSE,T.P.H. AND TAYLOR,J.M. (1977)
J.BIOL.CHEM., 252, 1272

TUSZYNSKI,G.P.,KNIGHT,L.,PIPERNO,J.R., AND WALSH,P.N. (1980)
ANAL.BIOCHEM., 106,118

Van DIEIJEN,G.,Van Der LAKEN,C.S., et al. (1975)
J.MOL.BIOL., 93, 35

VAN VENROOIJ,W.J. AND JANSSEN,D.B. (1978)
MOL.BIOL.REP., 4, 3

VÁZQUEZ,D. (1974)
FEBS.LETT., 40, S63

VENEBLES,P.J.W.,YI,T.,WOODROW,D.F.,MOSS,P., et al. (1983)
ANN.RHEUM.DIS., 42, 17

VINCENT,A.,GOLDENBERG,S., AND SCHERRER,K. (1981)
EUR.J.BIOCHEM., 114, 179

WAELTI,E.R. AND HESS,M.W. (1980)
J.IMMUNOL.METH., 32, 177

WANG,A.H.J.,QUIGLEY,G.J.,KOLPAK,F.J., et al. (1981)
SCIENCE, 211, 171

WASICEK,C.A. AND REICHLIN,M. (1982)
J.CLIN.INVEST., 69, 835

WASSERMAN,M.,ZAKAI,N.,LOYTER,A., AND KULKA,R.G. (1976)
CELL, 7, 551

WATANABE,Y.,IGARASHI,K., AND HIROSE,S. (1981)
BIOCHEM.BIOPHYS.ACTA., 656, 134

WEBER,L.A.,HICKEY,E.D., AND BAGLIONI,C. (1978)
J.BIOL.CHEM., 253, 178

WEBER,L.A.,HICKEY,E.D.,MARONEY,P.A., AND BANGLIONI,C. (1977)
J.BIOL.CHEM., 252, 4007

WEINSTEIN,J.N.,YOSHIKAMI,S.,HENKART,P., et al. (1977)
SCIENCE, 195, 489

WEISBROD,S. AND WEINTRAUB,H. (1979)
PROC.NATL.ACAD.SCI.USA., 76, 630

WEISSMAN,G.,COHEN,C., AND HOFFSTEINS,S. (1977)
BIOCHEM.BIOPHYS.ACTA., 498, 375

WELLS,A.F.,MILLER,C.E., AND NADEL,M.K. (1966)
APPL.MICROBIOL., 14, 271

WHITE,P.J.BILLINGS,P.B., AND HOCH,S.O. (1982)
J.IMMUNOL., 128, 2751

WHITE,P.J.,GARDNER,W.D., AND HOCH,S.O. (1981)
PROC.NATL.ACAD.SCI.USA., 78, 626

WHITE,P.J. AND HOCH,S.O. (1981)
BIOCHEM.BIOPHYS.RES.COMM., 102, 365

WIEBEN,E.D.,MADORE,S.J., AND PEDERSON,T. (1983)
PROC.NATL.ACAD.SCI.USA., 80, 1217

WILLIAMS,R.C. (1982)
ARTH.RHEUM., 25, 810

WILSON,M.R.,AMOYAVE,C.M.,MILES,L., et al. (1977)
AM.RHEUM.DIS., 36, 540

WILSON,T.,PAPAHADJOPOULOS,D., AND TABER,R. (1979)
CELL, 17, 77

WILSON,M.,SAWICKI,S.,SALDITT-GEORGIEFF,M., et al. (1978)
J.VIROL., 25, 97

WINCHESTER,R.J. (1978)
ARTH.RHEUM.,21,51

WINFIELD,J.B.,BRUNNER,C.M., AND KOFFLER,D. (1978)
ARTH.RHEUM., 21, 289

WINFIELD,J.B.,KOFFLER,D., AND KUNKEL,H.G. (1975)
J.CLIN.INVEST., 56, 563

WINN,D.M.,WOLFE,J.F.,LINDBERG,P.A., et al. (1979)
ARTH.RHEUM., 22, 1334

WISE,J.A. AND WEINER,A.M. (1980)
CELL, 22, 109

WODNAR-FILIPOWICZ,A.,SZCZESNA,E., et al. (1978)
EUR.J.BIOCHEM., 92, 69

WOODLAND,H.R.,GURDON,J.B., AND LINGREL,J.B. (1974)
DEVL.BIOL., 39, 134

WOOLEY,J.C.,ZUKERBERG,L.R., AND CHUNG,S-Y. (1983)
PROC.NATL.ACAD.SCI.USA., 80, 5208

WU,J.M. (1980)
FEBS.LETT., 110, 297

WU,J.M. (1981)
FEBS.LETT., 133, 107

YANG,V.W.,LERNER,M.R.,STEITZ,J.A., AND FLINT,S.J. (1981)
PROC.NATL.ACAD.SCI.USA., 78, 1371

YOSHIKI,T.,MELLORS,R.C.,STRAND,M., et al. (1974)
J.EXP.MED., 140, 1011

ZEEVI,M.,NEVINS,J.R., AND DARNELL,J.E. (1982)
MOL.CELL.BIOL., 2, 517

ZEHAVI-WILLNER,T. (1975)
FED.PROC.FED.AM.SOC.EXP.BIOL., 34, 706

ZEHAVI-WILLNER,T. AND POSTKA,S. (1976)
ARCH.BIOCHEM.BIOPHYS., 172, 706

ZIEVE,G.W(1981)
CELL, 25, 296

Appendix

The value of the incorporation of radioactivity into protein was determined by using duplicate samples, and only the average value is shown on Fig. 4.2.b, 4.3., 4.4., 4.6., 4.7., 4.8., 5.1., 5.4.a, 5.5.a, 5.6.a and b, 5.7., 5.10.a , 5.11.a, 5.15.a.

When comparing the ANA's with either cycloheximide or ATA inhibition, it is important to note that a single concentration (50μM) of each inhibitor was used. It may be that the shapes of the curves (Fig. 5.6a and b) and their position relative to each other will be different at higher or lower inhibitor concentrations. Thus, the conclusions drawn in the body of the thesis may likewise be changed. It must be emphasised that such an approach would be prohibitively expensive in terms of isotopes and cell-free lysates and is, at present, not possible.

The fundamental cell-free translation assays in this work are either wheat germ or reticulocyte lysates. While comparisons within each of these assays are of course absolutely valid, there are problems in making quantitative comparisons between the two systems. For example, comparing the absolute efficiency of translation is qualitative and therefore subject to differing interpretations (eg. Fig. 4.5.b).

Quantitation of the immunological effects is further exacerbated by differences in the data for normal individuals (eg. Ig stimulation of translation in Table 5.2.), where stimulation of translation seems to be the rule. It might be that clearer conclusions could have been drawn if more inhibitory/abnormal sera had been used and a comparison made with groups rather than individuals. A further, and practically insoluble, problem is that inhibition (antiRo,Frayne) or stimulation (normal IgG) of translation may be affected by the drugs taken by the patient, or indeed by the normals. The protein A purification (p.95) of the IgG's should have removed any of these interferring drugs, RNase or protease but for future work this needs to be proved beyond doubt.